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# The Composition of Pancreatic Juice as Compared to Sweat, Parotid Saliva and Tears.

By

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The ducts or tubules of glands with external secretion are usually quite complex in structure, and they differ morphologically to a considerable extent from gland to gland. "However, very little is known as to what part, if any, they play in the process of secretion, and few investigations dealing with this problem have been reported. Nevertheless, it would be quite wrong to make no attempt to understand the functions of the various ducts and to regard them solely as the pathways for the secretion formed in the alveoli" (BABKIN 1950).

MAXIMOW (1901), PISCHINGER (1924), RAWLINSON (1935) and several others have demonstrated that histological changes occur in the ducts of the salivary glands, when the glands are stimulated to secrete. These authors take their findings as an indication that the ducts do, somehow, participate in the secretory process. Possibly, therefore, the glandular tubules — like those of the glomerular nephron — may withdraw from or add to the secretion certain components and in this way contribute to the elaboration of the final secretory product. Physiological evidence in support of such hypothesis is, however, very scanty and in this respect the development of glandular physiology has fallen behind the progress made in our understanding of the functions of the uriniferous ducts of the glomerular kidney within the past thirty years.

The present paper represents an attempt at elucidating the possible functions of the glandular ducts by a comparative study of functional behaviour and structural appearance of the sweat, parotid, lacrymal and pancreatic glands. The experimental part of the paper is devoted to a study of the composition of canine pancreatic juice, obtained in response to secretin. The main purpose was to collect exact information on the variations in the concentration of sodium, potassium, chloride and bicarbonate in relation to secretory rate and to compare the results to similar investigations on the composition of human parotid saliva (THAYSEN, THORN and SCHWARTZ 1954), tears (THAYSEN and THORN 1954) and sweat (SCHWARTZ and THAYSEN 1956). The hypothesis is forwarded that sodium is being reabsorbed from precursor secretions in the striated ducts of the parotid gland and in the duct of the sweat gland, whereas a similar mechanism of sodium reabsorption cannot be demonstrated in the lacrymal and pancreatic glands which are devoid of striated epithelium.

## Procedure.

Large dogs, weighing about 20 kg, were used for the experiments. The dogs were anaesthetized with nembutal and sacrificed at the end of the experiment.

*Collection of pancreatic juice:* The duodenum was reached through an upper mid-line incision and a duodenotomy performed opposite to the mesentery. A cannula was inserted in the main pancreatic duct and held in position by a ligature in the duodenal mucosa. The secretory activity of the pancreatic gland was stimulated by the intravenous injection of 4 mg of secretin,[1] which caused a marked increase in secretory rate of 50 to 80 minutes' duration. The pancreatic secretion was collected in glass syringes of 1 to 5 ml capacity. A piece of fine plastic tubing, 3 to 4 cm long, served as a connection between the cannula and the tip of the syringe. At the end of each collection period the syringe was removed, immediately closed with a metal cap and stored in the cold (0 centigrades) until analysed. The "dead space" of the collection system from tip of syringe to tip of cannula was about 0.05 ml.

Collections of pancreatic juice were made in this way at accurately timed intervals of from 1 to 45 minutes' duration, in order to determine the secretory rate at various intensities of stimulation and under "resting" conditions. The largest stimulated flows were about 2 ml per minute. The "resting" flows were usually around 0.02 to 0.03 ml per minute. Approximately 0.8 to 4.0 ml of juice was collected in each

[1] The secretin-preparation used in the present experiments was kindly supplied by Professor Dr. E. JORPES, Karolinska Institutet, Stockholm, Sweden.

period. 10 to 15 individual periods were sampled following a single stimulation.

Venous blood was drawn into heparinized tubes at least twice during each experiment and separated by centrifugation for chemical analysis of the plasma.

*Determination of sodium and of potassium* in pancreatic juice and in plasma was carried out on a Perkin Elmer flame photometer (model 52 C), using lithium as an internal standard. The coefficients of variation for 10 replicate analyses for sodium and potassium on the same plasma were 3 and 2 per cent respectively.

*Determination of chloride* in pancreatic juice and in plasma was carried out by BRUN's (1949) modification of the method of SCHALES and SCHALES (1941). The coefficient of variation for 10 replicate analyses on the same plasma was 4 per cent.

*Determination of pH:* The pH of the pancreatic juice was determined within 3 hours of the collection. The samples had been stored in the cold in the manner mentioned above till immediately before they were analysed. pH was measured with a glass electrode at 38 centigrades under anaerobic conditions by the method of ASTRUP (1955). The accuracy of the determinations is within 0.005 pH units (ASTRUP 1955).

*Determination of "total $CO_2$"*: Total $CO_2$ in pancreatic juice was measured within 3 hours of collection on a VAN SLYKE machine by a standard procedure (PETERS and VAN SLYKE 1932). The coefficient of variation for 5 replicate analyses of the same sample was 4.5 per cent.

*The concentration of bicarbonate* in plasma was measured as "standard bicarbonate" at a $CO_2$ tension of 40 mm Hg and at 38 centigrades by the method of ASTRUP (1955).

*Determination of urea* in pancreatic juice and in plasma was carried out by the colorimetric method of ARCHIBALD (1945). The coefficients of variation for replicate analyses for urea in pancreatic juice and in plasma were 3 and 4 per cent respectively. It can be calculated from the results of these replicate analyses that a variation of about 6 per cent may occur in the secretion/plasma concentration ratio for urea merely due to technical errors.

*Determination of reducing sugar* in pancreatic juice and in plasma was carried out by the method of HAGEDORN and NORMAN-JENSEN (1923).

## Results.

*Excretion of sodium:* The concentration of sodium was measured in 39 samples of pancreatic juice, obtained in 3 experiments on 2 dogs. The secretory rates ranged from 0.02 to 2.0 ml per minute and the mean concentration of sodium in the 5 plasma samples drawn during the experiments, $\overline{PL_{Na}}$, was 152 ($\pm$ 2) mEq per liter.

Figure 1 shows that the concentration of sodium in the pancreatic juice is independent of a 100-fold range of variation in secretory rate.

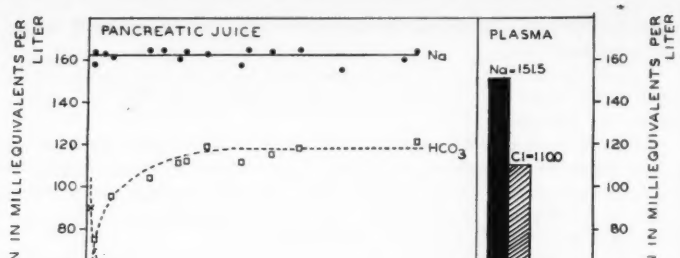


Fig. 1. Relation between the rate of secretion and the concentrations of sodium, potassium, chloride and bicarbonate in the pancreatic juice of dog A.

The mean concentration of sodium in all 39 samples of pancreatic juice, $\overline{PA_{Na}}$, was 161 ($\pm$ 4) mEq per liter. The coefficient of variation, 2.5 per cent, is of the same order of magnitude as the coefficient of variation for replicate analyses by the same method (3 per cent). No physiological variance of $PA_{Na}$ could thus be detected by the technique employed in the present experiments.

The mean secretion/plasma concentration ratio for sodium, $\overline{PA_{Na}/PL_{Na}}$, was 1.06. However, if the difference in the concentrations of total solids in pancreatic juice and plasma is corrected for, the ratio of the concentration of sodium in secretion water to the concentration of sodium in plasma water is found to be very close to unity.

*Excretion of potassium:* The concentration of potassium in the pancreatic juice was measured in the same 39 samples as was sodium. The mean concentration of potassium in the 5 plasma samples drawn during the experiments, $\overline{PL_K}$, was 5.28 ($\pm$ 0.31) mEq per liter.

Figure 1 shows that the concentration of potassium in pancreatic juice is apparently independent of a 100-fold range of variation in secretory rate.

The mean concentration of potassium in all 39 samples of pancreatic juice, $\overline{PA_K}$, was 5.33 ($\pm$ 1.08) mEq per liter. The coefficient of variation, 20 per cent, thus far exceeded the coefficient of variation for replicate analyses by the same method (2 per cent). The mean concentration of potassium in the pancreatic juice of dog A was 4.71 ($\pm$ 0.56) mEq per liter and of dog B 5.75 ($\pm$ 1.18) mEq per liter. This difference in potassium concentration was found to be significant by a t-test ($p < 0.01$). The demonstrated difference between dogs could, however, not account for more than a fraction of the total physiological variance of $PA_K$. The reason for the remaining physiological variance remains unaccounted for in the present experiments.

The mean secretion/plasma concentration ratio for potassium, $\overline{PA_K/PL_K}$, was 1.01.

*Excretion of bicarbonate:* The concentration of bicarbonate was measured in 33 of the 39 samples that had been analysed for sodium and potassium. The secretory rate ranged from 0.02 to 2.0 ml per minute and the mean concentration of bicarbonate in the 5 plasma samples drawn during the experiments was 25.5 ($\pm$ 2.0) mEq per liter.

The concentration of bicarbonate in the pancreatic juice was independent of secretory rate at flows exceeding 0.4—0.5 ml per minute. In dog A the concentration was approximately 120 mEq per liter, in dog B approximately 85 mEq per liter at the sesecretory rates.

At secretory rates below 0.4—0.5 ml per minute the concentration of bicarbonate gradually dropped and reached (in dog B) levels lower than the concentration of bicarbonate in the plasma.

*Excretion of chloride:* The concentration of chloride was determined in 36 of the 39 samples that had been analysed for sodium and potassium. 33 of the 36 samples analysed for chloride had in addition been analysed for bicarbonate. The secretory rates ranged from 0.02 to 2.0 ml per minute and the mean concentration of chloride in the 5 plasma samples drawn during the experiments was 110.7 ($\pm$ 4) mEq per liter.

The concentration of chloride in the pancreatic juice was found to vary inversely to the concentration of bicarbonate (see figure 1).

The sum of the concentrations of bicarbonate and chloride amounted to 87 ($\pm$ 4.7) per cent of the sum of the concentrations of the two cations at all secretory rates.

*The pH of the pancreatic juice:* In 7 samples of pancreatic juice obtained from dog A and in 16 samples obtained from dog B at secretory rates exceeding 0.4 ml per minute the mean values for pH were 8.11 ($\pm$ 0.07) and 8.04 ($\pm$ 0.08) respectively. At lower secretory rates pH gradually dropped to values equal to or lower than those of the plasma.

*The excretion of urea.* The concentration of urea was measured in 36 samples of pancreatic juice obtained from two dogs. The secretory rates ranged from 0.09 to 2.0 ml per minute and the concentrations of urea in the plasma of the two dogs were 18.7 and 21.8 mg per cent, respectively.

The concentration of urea in the pancreatic juice was independent of secretory rate. The mean ratio of the concentration of urea in the pancreatic juice to the concentration of urea in the plasma, $\overline{PA_u/PL_u}$, was 0.82 ($\pm$ 0.23). The coefficient of variation for $\overline{PA_u/PL_u}$ (29 per cent) thus exceeds the coefficient of variation to be expected from mere technical errors (6 per cent). The mean $\overline{PA_u/PL_u}$ ratio of dog A was 0.92 ($\pm$ 0.09) and of dog B 0.75 ($\pm$ 0.14), a difference which is found to be highly significant by a t-test ($p < 0.01$). However, the demonstrated difference between dogs only accounts for a fraction of the total physiological variance of $\overline{PA_u/PL_u}$. The remaining physiological variance remains unaccounted for in the present experiments.

*The excretion of reducing substance:* The concentration of reducing substance was measured in 5 samples of pancreatic juice with secretory rates ranging from 0.56 to 2.0 ml per minute. The average concentration of reducing substance in the 5 samples was 19.6 ($\pm$ 3.7) mg per 100 ml. The plasma concentration was determined twice and found to be 92 and 96 mg per 100 ml. The ratio of the average concentration of reducing substance in the pancreatic juice to the average concentration of reducing substance in the plasma, $\overline{PA_r/PL_r}$, was thus 0.21.

An intravenous injection of 50 ml of a 50 per cent solution of glucose was now given and the concentration of reducing substance followed during 1 hour in a further 6 samples of plasma and 7 samples of pancreatic juice. It can be seen from figure 2 that the six-fold increase in the concentration of reducing substance in the plasma caused by the injection of glucose is followed by a similar increase in the concentration of reducing substance in the pancreatic juice (from 19.6 to 119 mg per 100 ml).

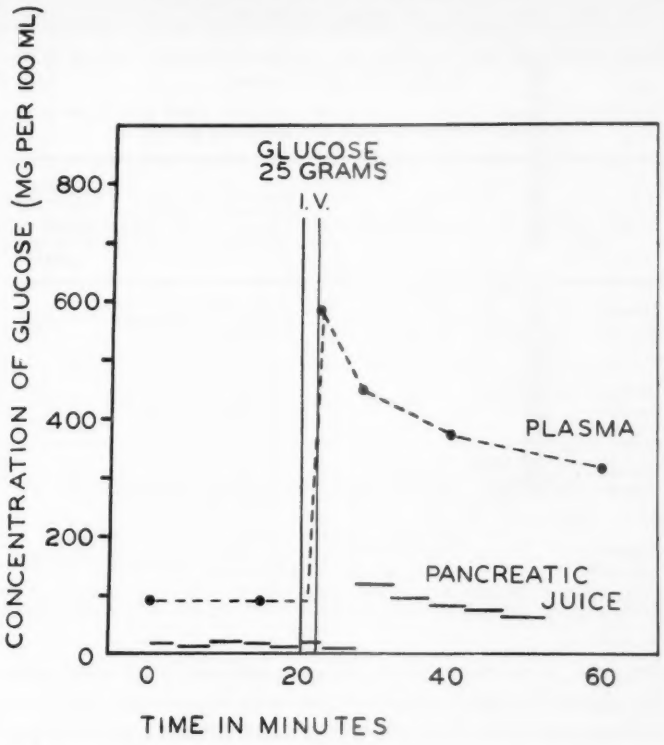


Fig. 2. Relation between the concentration of reducing substance in the plasma and in the pancreatic juice before and after an intravenous injection of glucose.

## Discussion.

*The Excretion of Electrolytes.*

On the whole the present experiments on the composition of pancreatic juice confirm the work of previous authors (GAMBLE and MCIVER 1928, BALL 1930, KOMAROV, LANGSTROTH and MCRAE 1939, LAGERLÖF 1942, HART and THOMAS 1945). The findings have, however, been somewhat extended by the simultaneous determination of the different electrolyte species in almost every sample of pancreatic juice and by the fact that many samples, secreted over a very large range of variation in secre-

**Table 1.**

*Range of secretory rate in milligrams per gram gland per minute of the different glands examined.*

Calculations of secretory rates in milligrams per gram gland per minute of the sweat, parotid, lacrymal and pancreatic glands.

| Gland | Reference | Mode of stimulation | Range of secretory rates observed | Weight of gland | Secretory rate in mg per gram gland per minute |
|---|---|---|---|---|---|
| Human parotid | THAYSEN, THORN & SCHWARTZ 1954 | "Mecholyl" 4—6 mg subcutaneously | 0.2—4.26 ml/minute | 30 grams | 7—140 |
| Human lacrymal | THAYSEN & THORN 1954 | Onions | 10—121 mg/minute | 1.5 grams | 7—80 |
| Human sweat | SCHWARTZ & THAYSEN 1956 | "Mecholyl" 1 mg intradermally | 0.1—2.0 mg/cm²/min | 20 mg/cm² | 5—100 |
| Canine pancreatic | | Secretin 4 mg intravenously | 0.02—2.00 ml/minute | 25 grams | 0.8—80 |

tory rate, were obtained in each experiment. This is of great importance if prime emphasis is placed on the possible variations in the composition of a secretory product with the rate of secretion, and was aimed at here for the explicit purpose of a comparison of pancreatic juice with previous similar studies on parotid saliva, tears and sweat. In figure 3 a comparison is made between these four secretory products with respect to the excretion of sodium, potassium, chloride and bicarbonate. The concentrations of the different electrolytes are plotted against secretory rate, expressed in milligrams per gram glandular tissue per minute. The secretory rates per unit weight of gland have been calculated as demonstrated in table 1. The combined mass of the sweat glands within 1 cm² of skin has been estimated from the glandular dimensions, assuming that the specific gravity of the tissue is 1 and that the skin on the ventral surface of the forearm contains an average of 100 individual glands (DOLE and THAYSEN 1953).

The following similarities and differences between the four secretory products are apparent from figure 3:

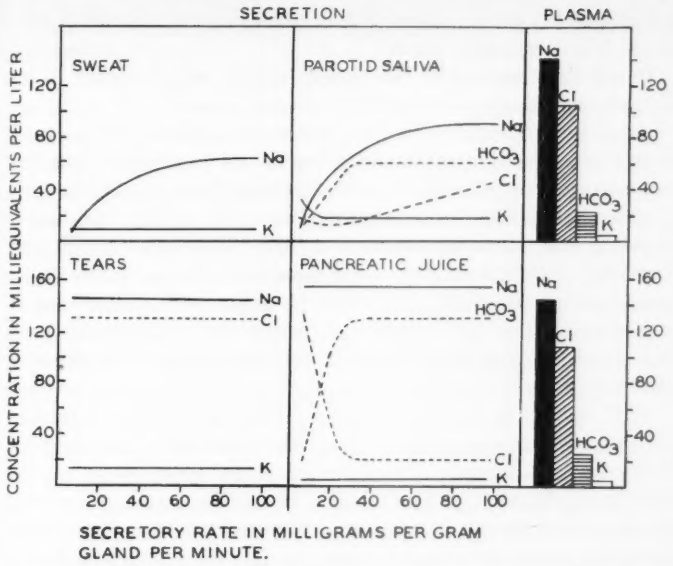


Fig. 3. Relation between electrolyte concentration and secretory rate (in milligrams per gram gland per minute) of the sweat, parotid, lacrymal and pancreatic glands.

*The Excretion of Sodium:*

In sweat and in parotid saliva the concentration of sodium is always smaller than the concentration of sodium in the plasma. In both secretions the concentration of sodium varies with secretory rate. With increasing secretory rate the concentration of sodium rises from approximately 5 mEq per liter in both secretions to about 60 mEq per liter in the sweat and about 90 mEq per liter in parotid saliva.

In tears and in pancreatic juice the concentration of sodium is approximately equal to the concentration of sodium in plasma water. In both secretions the concentration of sodium is independent of large variations in secretory rate.

*The Excretion of Potassium:*

The concentration of potassium in all four secretions is equal to or greater than the concentration of potassium in the plasma. Thus, the $\frac{\text{secretion}}{\text{plasma}}$ concentration ratio for potassium is about

4.5 in parotid saliva, about 3.5 in tears, about 2 in sweat and about 1 in pancreatic juice.

In all four secretions the concentration of potassium is independent of large variations in secretory rate.

However, in parotid saliva an increase in potassium concentration is noted at secretory rates below 0.5 ml per minute, and the concentration of potassium in the "resting" (unstimulated) secretion is always significantly greater than that of the stimulated secretion (LANGSTROTH and MCRAE 1938, THAYSEN, THORN and SCHWARTZ 1954). A similar rise in potassium concentration in the unstimulated pancreatic juice can be ruled out by the present experiments. The concentration of potassium in pancreatic juice was found to be independent of a 100-fold range of variation in secretory rate.

With respect to the two other secretions no definite statement can be made at the present time. The tears were examined by THAYSEN and THORN over a 12-fold range of variation in the rate of lacrimation and no rise in potassium concentration was observed at low secretory rates. However, "resting" secretory rates can hardly be obtained when the tear fluid is collected on a foreign body placed in the conjunctival sack. As regards the sweat the method of collection, used by SCHWARTZ and THAYSEN (1956), hardly permits any observations on possible variations in potassium concentration at the very low rates of secretion. (It is not possible to obtain, in the individual experiment, many samples of sweat secreted over a wide range of variation in secretory rate.) It remains for future experiments to decide how potassium is excreted in tears and in sweat at low secretory rates. It is tempting to assume that the sweat gland is comparable to the parotid gland and the tear gland comparable to the pancreatic gland not only with respect to sodium excretion, but also with respect to the excretion of the potassium ion.

*The Excretion of Anions:*

In sweat and tears chloride is the main anion. Chloride concentration of sweat is not depicted in fig. 3, but according to LOCKE, TALBOTT, JONES and WORCESTER (1951) chloride concentration of sweat is slightly smaller than the sum of the concentration of sodium and potassium at all secretory rates. THAYSEN and THORN (1954) found chloride concentration of the tears to

equal 80 per cent of the sum of the concentrations of sodium and potassium.

In parotid saliva and in pancreatic juice chloride is the main anion at low secretory rates. With increasing secretory rate the concentration of bicarbonate rises in both secretions, reaching a maximum of about 60 mEq per liter in parotid saliva and about 90—130 mEq per liter in pancreatic juice. When this maximum concentration has been arrived at the concentration of bicarbonate is independent of further increases in secretory rate in both secretions. The concentration of chloride varies inversely with that of bicarbonate in both secretions. In parotid saliva as well as in pancreatic juice the sum of the concentrations of chloride and bicarbonate is equal to about 90 per cent of the sum of the concentrations of sodium and potassium at all secretory rates.

It will be noted from the above comparison that the excretion of sodium in sweat and parotid saliva is distinctly different from sodium excretion in tears and pancreatic juice. The sweat gland and the parotid gland appear to possess a sodium conserving mechanism of a limited maximal capacity. A hypothetical explanation of the nature of this sodium conserving mechanism is attempted on the basis of the findings illustrated in figure 4. In the bottom part of this figure the rate of excretion of sodium (in $\mu$ Eq per gram gland per minute) is plotted against secretory rate (in mg per gram gland per minute) for all four glands in question. It can be seen that the rate of excretion of sodium is a linear function of secretory rate in tears and pancreatic juice. The calculated regressions show a small, and statistically insignificant, positive intercept. In sweat and parotid saliva, however, the rate of sodium excretion approaches a linear function of secretory rate only when the latter exceeds 30 mg per gram gland per minute. The calculated regressions for all data at or above secretory rates of 30 mg per gram gland per minute show statistically significant negative intercepts of $-$ 1.47 ($\pm$ 0.24) $\mu$ Eq per gram gland per minute in the case of sweat and $-$ 1.96 ($\pm$ 0.11) $\mu$ Eq per gram gland per minute in the case of parotid saliva. As suggested by THAYSEN, THORN and SCHWARTZ (1954) and SCHWARTZ and THAYSEN (1956) these findings are compatible with the assumption that sodium is delivered into a precursor secretion at the rate of 0.079 $\mu$ Eq per mg of sweat and of 0.106 $\mu$ Eq per mg of parotid saliva, and that sodium is being reabsorbed

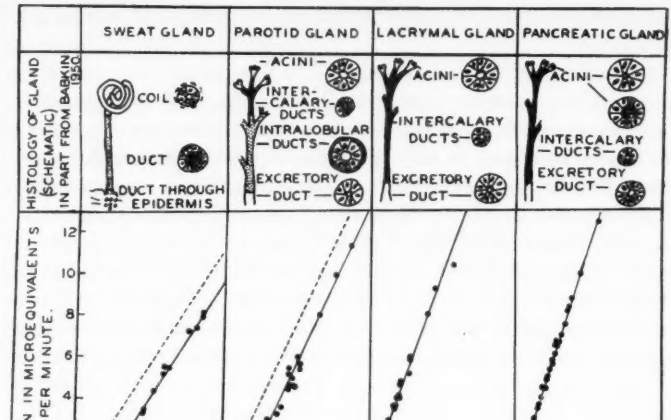


Fig. 4. Relation between histological structure and sodium excretion (in microequivalents per gram gland per minute) of the sweat, parotid, lacrymal and pancreatic glands.

from these precursor secretions at the rates 1.47 and 1.96 $\mu$ Eq per gram gland per minute, respectively. In contrast, sodium does not appear to be reabsorbed from the precursor of tears (THAYSEN and THORN 1954) nor from the precursor of pancreatic juice. Seemingly, sodium concentration is lower in the precursor secretions of sweat (79 mEq/l) and parotid saliva (106 mEq/l) than in the precursors of tears (136 mEq/l) and pancreatic juice (159 mEq/l). It is, however, not possible to estimate sodium concentration of the precursor secretions nor the absolute amount of sodium being reabsorbed as long as the direction and amount of water movement within the secreting glands remain obscure.

Assuming that the above theory is correct, it appears reasonable to suggest that the formation of the precursor secretion and the subsequent reabsorption of sodium occur in morphologically distinct parts of the glands. A comparison between the histological

structure of the parotid, lacrymal and pancreatic glands (as depicted schematically in figure 4) shows that all glands possess acini, intercalary ducts and excretory ducts and that the morphology of these structures is closely similar in the three glands. In contradistinction to the two other glands, the parotid gland, moreover, possesses striated ducts which are interposed between the intercalary and the excretory ducts. The main morphological difference between the three glands is, thus, the presence of striated ducts in the parotid gland and the lack of this structure in the two other glands. The main functional difference between the three glands, according to the above mentioned hypothesis, is that sodium is being reabsorbed from the precursor of parotid saliva but not from the precursors of tears and pancreatic juice. It is tempting, therefore, to suggest that the hypothetical sodium reabsorption in the parotid gland takes place in the striated ducts. As regards the sweat gland, one may assume that sodium reabsorption takes place in the double layered epithelium of the duct (SCHWARTZ and THAYSEN 1956).

It is of some interest in this connection to study the work of MERKEL (1883) and WERTHER (1886). MERKEL suggested that the striated ducts of the salivary glands secrete the salts of the saliva, drawing a parallel to the then current concept of the functions of the striated epithelium in the proximal tubules of the nephron. WERTHER subjected this hypothesis to experimental investigation, but found, contrary to expectations, that the concentration of salt in different salivas was inversely related to the amount of striated epithelium in the ducts of the corresponding glands. Saliva from glands which are completely or almost completely devoid of striated ducts (rabbits parotid and canine sublingual) is isotonic with the plasma, whereas saliva from glands with ample striated epithelium (canine parotid or submaxillary) is always hypotonic as compared to plasma. WERTHER apparently gave up further investigation of the matter. In the light of modern concepts of kidney physiology WERTHER's experiments may, however, be taken to indicate that the striated ducts of the salivary glands, like the striated epithelium in the proximal tubules of the kidney possess primarily reabsorptive functions. Sodium concentration in relation to secretory rate should be examined in salivas secreted by glands with varying amounts of striated epithelium in the ducts in order to reinvestigate the whole problem. Researches along these lines are further stimulated by the work of LUND-

BERG (1955), who by independent means (studying the electrophysiology of the submaxillary gland) has presented evidence in favour of the hypothesis that cations are reabsorbed by the striated epithelium.

*The Excretion of Reducing Sugar.*

It is demonstrated in the present experiments that pancreatic juice contains reducing substance at about 1/5 of the concentration of reducing substance in the plasma. Moreover, this secretion/plasma concentration ratio for reducing substance was found to be unaffected by a 5-fold increase in the concentration of reducing substance in the plasma, caused by the intravenous injection of glucose. GIARDINI and ROBERTS (1950) have shown that tears do, likewise, contain a considerable concentration of reducing sugar.

In contrast, sweat does not contain reducing sugar even when the concentration of reducing sugar in the blood is markedly increased as in diabetic coma or following intravenous injection of glucose (LOBITZ and OSTERBERG 1947). The same applies to saliva, obtained by nervous stimulation or following the injection of cholinergic drugs (LANGSTROTH, MCRAE and STAVRAKY 1938).

It, thus, appears that the distinction made above between the sweat and parotid glands on one side and the lacrymal and pancreatic glands on the other does not only apply to sodium but also to the excretion of glucose. The mechanism by which glucose is conserved by the sweat and parotid glands remains, however, even more obscure than the mechanism for sodium conservation. It would be of considerable interest in this connection to know whether the precursors of sweat and saliva contain glucose.

*The Excretion of Urea.*

The secretion/plasma concentration ratio for urea in pancreatic juice averaged 0.8 in the present experiments and was independent of secretory rate. In human tears the ratio is, similarly, close to unity and independent of the rate of secretion (THAYSEN and THORN 1954).

In sweat and in unstimulated parotid saliva, however, the secretion/plasma concentration ratio for urea is considerably greater than unity, and it has been suggested that in these secretions urea is raised to concentrations greater than those of the plasma by reabsorption of water from precursor solutions formed

within the secreting glands (SCHWARTZ, THAYSEN and DOLE 1953, ALBRECTSEN and THAYSEN 1955).

Thus, with respect to urea excretion a further difference is noted, which distinguishes the sweat and parotid glands from the lacrymal and pancreatic. Whether the above hypothetical explanation of the mechanism of urea excretion by the sweat and parotid glands is correct can not be decided upon at present. However, even in case proof can be presented for the validity of the theory, it must be realized that measurement of the "clearance" of urea or any other substance by a gland can probably at most yield qualitative, but never quantitative information about precursor formation and water reabsorption. In contrast to what is the case in studies on the glomerular nephron, it is very unlikely that in glandular physiology one should be able to find substances to which the region of precursor formation is freely permeable and the region of water reabsorption completely impermeable. For the same reasons it must be realized that the finding of secretion/plasma concentration ratios for urea equal to or smaller than unity in the case of tears and pancreatic juice does not permit any conclusions regarding the existence or non-existence of an internal circulation of water in the lacrymal and pancreatic glands.

## Summary.

The concentrations of sodium, potassium, chloride, bicarbonate, glucose, urea and the pH of pancreatic juice, obtained in response to secretin, were examined in two dogs over a 100-fold range of variation in secretory rate. The concentrations of sodium and of potassium were 161 ($\pm$ 4) and 5.3 ($\pm$ 1.1) mEq per liter respectively and independent of secretory rate. The concentration of bicarbonate was independent of secretory rate at flows exceeding 0.5 ml per minute and averaged 120 mEq per liter in one of the dogs and 85 mEq per liter in the other. At secretory rates lower than 0.5 ml per minute the concentration of bicarbonate gradually dropped to values equal to or lower than plasma concentration. The concentration of chloride varied inversely with that of bicarbonate, the sum of the concentrations of the two anions amounting to 87 ($\pm$ 5) per cent of the sum of the concentrations of the two cations at all secretory rates. The pH averaged 8.05 at secretory rates exceeding 0.5 ml per minute. The secretion/plasma concentration ratio for urea was 0.9 and 0.8 in the two dogs and in-

dependent of secretory rate. The secretion/plasma concentration ratio for glucose was approximately 0.2 and independent of the concentration of glucose in the plasma.

The findings are compared to previous similar studies on human sweat, parotid saliva and tears. The hypothesis is forwarded that glandular secretion involves the formation of precursor solutions, which are modified by the reabsorption of sodium and of water in the ducts of the sweat and parotid glands, whereas similar reabsorptive processes cannot be demonstrated in the case of the lacrymal and pancreatic glands. Moreover, glucose appears to be conserved by the sweat and parotid glands, but not by the two other glands.

*Acknowledgement:* The skilful technical assistance of miss KAREN TYGSTRUP is gratefully acknowledged.

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# Tonic and Phasic Ventral Horn Cells Differentiated by Post-Tetanic Potentiation in Cat Extensors.

By

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Recently it was shown (GRANIT 1956 a) that, after tetanization of intact muscular afferents in decerebrate cats, the single-fibre stretch reflexes activated through the same afferents were potentiated. Instead of one or a few reflex spikes from the isolated motoneurones, there now followed a whole series of them, sometimes for as long as stretch was maintained. A state of spasticity — by definition an exaggerated stretch reflex — was thus created by tetanization, and further tests showed that a sequence of natural or adequate impulses had the same potentiating effect, provided that its frequency and duration were great enough for this purpose.

Now, since the gamma system by its activity causes the muscle spindles to discharge long-lasting series of impulses at high frequency, the motor neurones can be "labelled" by such spindle impulses and singled out for spastic behaviour or moderate facilitation, as the case may be. These facts raise a number of problems one of which will be dealt with in this paper.

When ventral horn cells have been made 'spastic' in this fashion, will they all behave in the same way or will some of them fail to respond iteratively? It was clear from the earlier

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experiments (GRANIT 1956 a) that potentiated motoneurones differed a great deal in their responses to stretch but more data seemed necessary for settling this question unequivocally. Therefore it was decided to explore in a preliminary survey the behaviour of 100 potentiated ventral horn cells (in ten animals) in order to establish whether fundamental or accidental differences accounted for the observations made. It is, of course, well known that in the non-potentiated state individual motoneurones respond very differently to stretch (GRANIT and STRÖM 1951, confirmed *e. g.* by ALVORD and FUORTES 1953).

This question may be restated in the following way: are the potentiated motoneurones which subserve long-lasting 'tonic' or postural motor functions (as determined by their stretch reflex to muscular afferents) different, within limits, from those that initiate phasic responses? We found this to be the case and proceed to present the evidence for this conclusion.

## Methods.

The methods were set out in detail in the preceding paper (GRANIT 1956 a) and will now be briefly summarized.

Cats, decerebrated by precollicular suction and de-efferented up to L5, were used. The gastrocnemius-soleus muscle was isolated and one pair of stimulating electrodes placed on the adjacent nerves *gastroc. med.* and *lat.* The other nerves to the leg were severed. The myograph was fixed on a sledge which, for testing by stretch, was pulled up by hand 10 mm against a stopper. Initial tension was near zero, in fact, adjusted so as to make the myograph barely respond to light touch of the string joining muscle and myograph. This amount of stretch will activate a large proportion of the cells capable of responding with a stretch reflex. Single efferent fibres were isolated by dividing ventral roots. Sometimes two or three active motoneurones remained, but in most cases individual fibres were obtained. For reasons to become clear below it is of considerable interest also to have some preparations with more than one active fibre.

Stretch and the reflex spikes were recorded vertically on running paper while a rapid horizontal sweep served for separation of spikes on a fast time base in order to make close inspection possible.

Every experiment began with three control stretches. Then followed tetanization of the muscular afferents at around 500/sec. for 10 sec. During tetanization the muscle was disconnected from the myograph and thus contracted against zero load. In this arrangement the muscle spindles are tetanized antidromically. It will last not less than 15—20 sec. before they are capable of responding normally (GRANIT 1956 a). Therefore the first test by stretch followed 20 sec. after cessation of

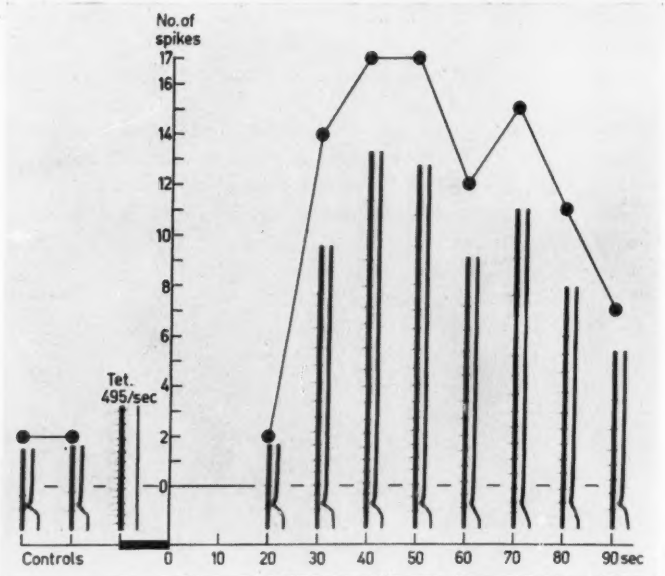


Fig. 1. Single ventral root fibre. Tonic type. Samples of original records of stretch reflexes inserted within graph in which ordinates show number of impulses delivered in response to stretch and abscissae time after tetanization period (black oblong), preceded by two observations on number of impulses in control stretch. Record taken during tetanization period inserted above black oblong. In this and successive figures the record during tetanization is cut to indicate time of 1 sec. Myograph record below stretch reflexes.

the tetanus, the following ones at intervals of 10 sec. In this manner the rise and fall of the post-tetanic potentiation was followed to the end by photographic recording. When no potentiation occurred the experiment could be carried out by inspection.

## Results.

1. *Two types of ventral horn cells.* Fig. 1 illustrates a ventral horn cell that during control stretch responded with two impulses. Tetanization brought out another ventral root spike, not activated by stretch. It is seen that after the tetanus the number of reflex spikes rose to 17, stretch being discontinued when no more impulses were heard in the loudspeaker. Thus the index of the effect is total number of spikes. Frequency is less adequate

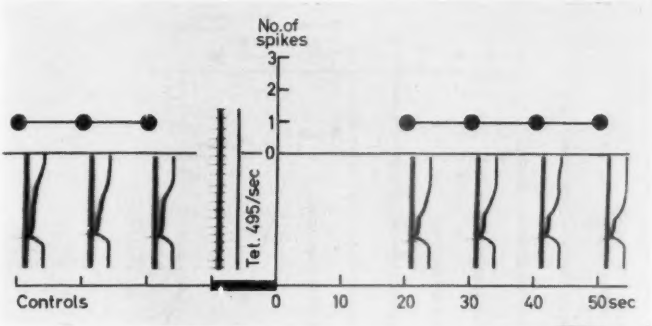


Fig. 2. Same as Fig. 1. Ventral horn cell of phasic type.

for the following reasons: (i) when there is one spike or a brief phasic discharge in the rising phase of control stretch, frequency is not an adequate basis of comparison of results before and after tetanization; (ii) frequency varied much less than the duration of the discharge which therefore is a better index when tonic effects are aimed at; (iii) it is well known from the work of ADRIAN and BRONK (1929), since repeatedly confirmed, that the frequency spectrum of ventral horn cells is exceedingly limited. Total number of spikes, however, may vary from zero to infinity. It is thus a good measure of the amount and duration of the post-tetanic effect. The variations of frequency may be studied in Figs. 1 and 4.

Another type of response, the phasic one, is illustrated in Fig. 2. The results are presented in the same manner as in Fig. 1. Clearly tetanization had no measurable effect whatsoever on this ventral horn cell. It was phasic and remained so.

The 100 spikes examined could be divided into three groups comprising phasic, moderately tonic and highly tonic cells but for the present purpose it suffices to divide them into the two main categories of phasic and tonic cells. Into the first group fell 53 % with very little or no potentiation. A reasonably good estimate of the type could be made already by inspecting the response to control stretch (before tetanization) because 88 % of the phasic cells only gave one spike or two closely spaced spikes on the rising phase of the pull, some of them none, while most of the tonic cells also responded to plateau stretch by a few impulses.

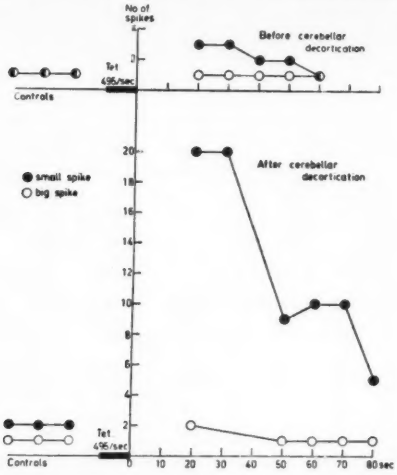


Fig. 3. Ventral root filament with two active fibres (small and large spike) showing their stretch reflexes (as in preceding figures) *before* (upper) and *after* (lower graph) decortication of cerebellar anterior lobe by suction.

Exceptions from this rule of thumb were found among the tonic cells some of which gave a brief phasic response in the controls (as *e. g.* the intensely tonic spike of Fig. 4). This is easily understood since, if for some reason or other excitability of the ventral horn cell is low or if autogenetic inhibition is present, post-tetanic potentiation will be needed to force the cell to sustained activity.

A few cells discharged permanently already to control stretch so that total number of spikes (our index) from the beginning was infinity. In these cases an effect of tetanization could be seen in the increased frequency of discharge. By our index these cells could not be classified.

Our material contained 3 % unclassified cells, 16 % cells intensely tonic after tetanization (a specimen in Fig. 4), 28 % tonic and, as stated, 53 % phasic cells.

This distribution cannot be held to be significant for an extensor population. To be sure, tonic cells were found in all cats but sometimes it was necessary to make a search for them. Phasic cells proved easier to find (see also below, section 3 on fibre size). The most important limiting condition would seem to be the state of the animal, *i. e.* amount of extensor tonus. Post-tetanic poten-

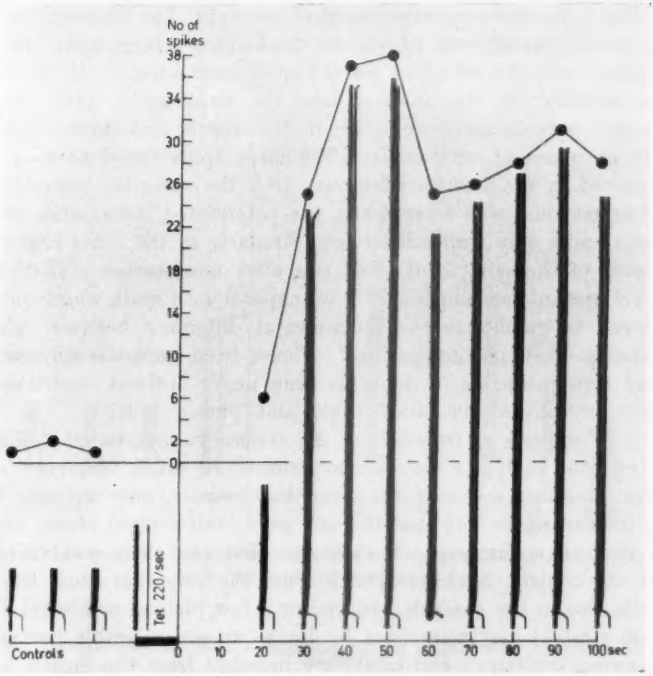


Fig. 4. As Fig. 1 but illustrating single-fibre stretch reflex in ventral horn cell showing intense potentiation. Initial phasic discharge not clearly visible at the reduced size of the records.

tiation is a presynaptic effect but it is tested with the aid of a post-synaptic index which requires a good level of excitability.

In order to throw light on this factor we had recourse to spinalization or decortication by suction of the anterior lobe of the cerebellum. In the spinalized state extensor tonus is well known to be low and accordingly it proved difficult to demonstrate the tonic response, despite potentiation. More important from the present point of view were the cerebellar ablations which create a state of high extensor tonus (BREMER 1922; STELLA 1944 a, b; MORUZZI 1950, GRANIT, HOLMGREN and MERTON 1955). Three such experiments were carried out. In them filaments tested before cerebellar decortication were set apart to be re-tested after the operation.

Fig. 3 illustrates an experiment of this type. The filament used contained two efferent fibres, one discharging a large spike from a phasic cell, the other one a small spike from a tonic cell. Before decortication of the anterior lobe the small spike gave very modest potentiation, two spikes in the control and three during the optimum of potentiation. The large spike failed to be influenced by the preceding tetanus. After the operation the small-spike response was 20 spikes in the potentiated state, while the large spike only responded twice. Similarly in the other experiments of the same kind there was after tetanization a shift of level upwards accompanied by an expansion of scale which only served to emphasize the fundamental difference between cells capable of supporting postural reflexes from muscular afferents and cells incapable of doing so even under optimal conditions, here for convenience called 'tonic' and 'phasic' cells.

2. *Frequency of tetanization.* An intensely potentiated cell is illustrated in Fig. 4 from an experiment in which frequency of the tetanizing stimulus was lower than usually, only 220/sec. It is interesting to note that this cell gave both a rapid phasic and a slow tonic discharge of which the first one alone was visible in the control. Such exceptions from the rule that most tonic cells also in the controls had one or a few plateau spikes (at 10 mm stretch) may sometimes be due to an unfavourable balance between excitatory and inhibitory impulses from the muscle receptors redressed by the facilitation in the potentiated state. By our criteria this cell is highly tonic.

Some systematic experiments on frequency of the tetanizing stimulus proved that beyond 200/sec. this factor was of little importance. The following figures are from a typical case: a cell which responded with two spikes to control stretch began to show definite potentiation with 5 spikes at stimulus rate 115/sec. for 10 sec.; at rate 130/sec. it gave 22 spikes, at 200/sec. 50 spikes, at 320/sec. the same number, which was maintained up to tetanizing frequencies around 500/sec. The agreement between our figure of 200/sec. and LLOYD's (1949) value of the same order with the monosynaptic response as index of potentiation may well be fortuitous. The monosynaptic index measures number of subliminal-fringe neurones added by potentiation, as tested by synchronous shocks. We are measuring total number of impulses of a single active neurone stimulated adequately by stretch.

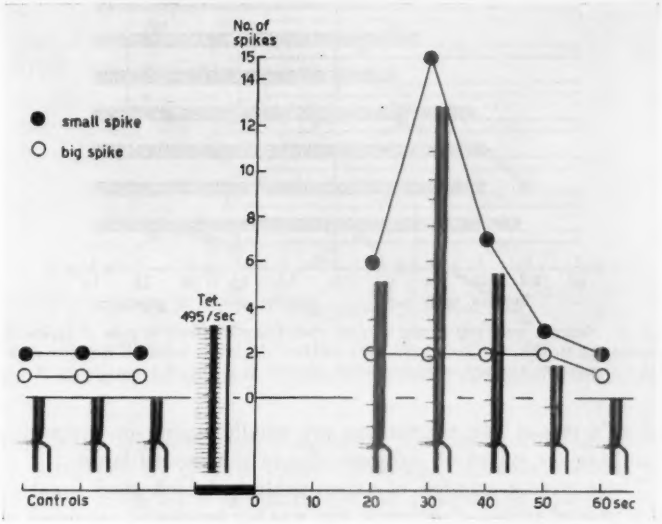


Fig. 5. As Fig. 1 with the difference only that the ventral root filament contained one large and one small spike.

3. *Significance of fibre size.* A common experience in these experiments was that when large and small spikes were present in the same filament, the large ones gave phasic and the small ones tonic types of response. This is shown in Fig. 5 (cf. also Fig. 3). While dissection of the filaments proceeded, we often at intervals tested their properties by tetanization and stretch in order to find out what kinds of spikes were left. Many such filaments were discarded because of unsuccessful isolation in the final stage, others became part of our collection. Discarded filaments did, however, enrich our material of observations on spike size and supported the generalization that tonic spikes tended to be among the small ones. In particular it was found that the largest spikes practically always were phasic.

Spikes will, of course, become small also when their fibres are killed below the point of recording but there is nothing to suggest that large tonic fibres would be more brittle than large phasic ones. Caution should be exercised in evaluating the size of spikes studied at different times of the day or in different animals and

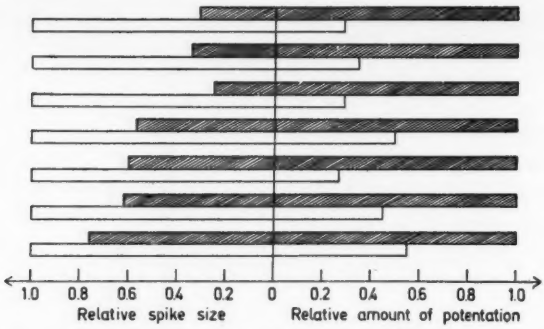


Fig. 6. Diagram referring to six ventral root filaments with a pair of spikes in response to stretch, one large, the other smaller, the latter hatched. Relative spike size and relative amount of potentiation plotted in terms of a maximum of 1.0.

for this reason our conclusions are wholly based on comparing simultaneous spikes of different size in the same filament.

There were a number of experiments in which good isolation of a pair of spikes of different size was the successful net result of dissection. These have been collected in the diagram of Fig. 6. The larger spike arbitrarily has been given size 1.0 and the maximum potentiation of the smaller spike similarly a potentiated value of 1.0. Relative size and relative amounts of potentiation have been plotted in terms of these maxima. It is seen that there is less potentiation in the larger spike of a pair.

Finally Fig. 7 is a plot of results with a filament in which three clearly discernible spikes were obtained. Their relative size is given in the inset, the main figure illustrating the amounts of potentiation obtained, which is seen to be less, the greater the spike size.

It is concluded that there is a definite tendency of the tonic cells to discharge spikes in smaller fibres than the phasic ones. The interpretation is that large cells are more likely to issue large fibres and hence that fibre size is an index of cell size. Thus the majority of the tonic cells would be grouped among the smaller alpha motor cells of the ventral horn.

4. *Spike frequency during tetanus.* If the failure on the part of the large cells to fire repetitively in response to stretch following tetanization were due to inhibition from muscular afferents or tendon organs, one would expect them to be more easily suppressed by tetanization, at least if Golgi tendon organs with

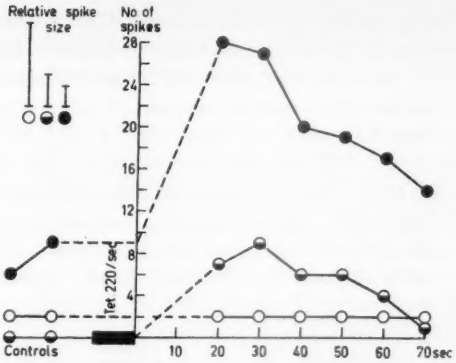


Fig. 7. As Fig. 5, but with three spikes in the filament. Their relative size shown.

their large afferents were responsible for the postulated effect (cf. discussion by GRANIT 1955). There was, however, nothing to indicate such a state of affairs. On the contrary, it was our impression that the large spikes ran up to higher frequencies during tetanization than the small ones. Samples of firing during tetanization are given in Figs. 1, 2, 4 and 5. Our results cannot very well be used to settle this point because other spikes than the ones responding to stretch turned up so often that evaluation of the whole material from this point of view proved difficult.

## Discussion.

The idea in this paper has been to use post-tetanic potentiation as a means for detecting fundamental categories of motoneurones (in their relationship to muscular afferents). Some indications of the two categories described may often be found without recourse to potentiation but a preceding tetanus, as it were, tears them apart by favouring repetitive firing and revealing latent tonic behaviour. Differences on a small scale are thus enormously magnified by potentiation and thereby the experiment becomes far less dependent on the state of the preparation. (Anaesthesia must be avoided in experiments on tonic cells because under the influence of almost any depressant drug all cells tend to become phasic (cf. ALVORD and FUORTES 1953). Chlorpromazine (HENATSCH and INGVAR 1956) and myanesin (GRANIT 1956 b) have the same effect as anaesthetics.)

One further advantage of potentiation is that this state by no means is unnatural. It was recently shown (GRANIT 1956 a) that the effect which here has been obtained by means of an electrical tetanus also occurs after natural stretch of sufficient intensity. This means that the gamma system will be of paramount importance for tonic cells because, activated by the brain stem (GRANIT and KAADA 1952, ELDRED, GRANIT and MERTON 1953, GRANIT and HOLMGREN 1955) this system may set up long-lasting states of intense activity from the muscle spindles succeeded by post-tetanic potentiation. The present work shows that the spindle loop acquires a different order of significance depending upon whether its influence is directed towards tonic or phasic cells. Only in the former case can it exercise its full effect on postural reflexes. The tonic cells, as it were, provide the *raison d'être* for the tonic gamma control from higher levels. At the same time the gamma control, on account of the presynaptic nature of potentiation, will label ventral horn cells over the spindle loop, as explained in the introduction. It is well known that spastic patients may appear normal, unless tested by stretch, when a potentiated state of their ventral horn cells is revealed.

The amount of stretch is a factor of considerable importance in that the tonic motoneurones on the whole seem to require less tension (elongation of muscle spindles) than, at least, the largest phasic ones. In the previous paper (GRANIT 1956 a) low tension (= small amounts of stretch) was used in an attempt to avoid reflex spikes in the controls and demonstrate potentiation by the sudden appearance of a tonic discharge to stretch after tetanization. It is, however, a problem in its own right, as yet wholly unsolved, to elucidate the difference between tonic and phasic ventral horn cells from the point of view of the variations in afferent input.

It is not necessary to overemphasize the correlations between, on the one hand, phasic and tonic motoneurones, on the other, their relative spike sizes from which we have inferred that the tonic neurones tend to group themselves among the smaller alpha ventral horn cells. Such biological classifications generally tend to go with a considerable region of overlap and transitional forms. This, however, does not detract from their significance.

Though not found in mammals, subdivision of ventral horn cells into motoneurones for phasic and tonic reflexes may well explain some amphibian results (BREMER and MOLDAVER 1934).

In their work a fast phasic reflex contraction was followed by a slow tonic one but no steps were taken to prove by dissection that different motoneurones were concerned.

In a recent paper NORRIS and GASTEIGER (1955) used fine microelectrodes for discrete responses from human muscles in voluntary contraction. There were four classes of spike amplitudes. The extremes were spikes of amplitude 0.63 and 2.47 mV. The small spike turned up first with weak voluntary contractions and increased its rate of firing a great deal as strength of contraction increased. The big spike required very strong contractions and fired less than 5 spikes in occasional bursts. The amplitudes of spikes, intermediate in size, represented motoneurones with properties transitional between the extremes. Since it is unknown how these muscle spikes are related to fibre size in the motor nerves, the results at the moment are difficult to compare with ours, though suggestive as far as they go. The work of NORRIS and GASTEIGER should be repeated in animals using post-tetanic potentiation to test the stability of the discharge properties of the muscle fibres isolated by such means. Ordinary myography would seem to be too coarse a method for this purpose. Also there is a considerable amount of overlapping or multiple innervation (HUNT and KUFFLER 1954) of individual muscle fibres which means that tonic and phasic fibres may run to the same motor unit. In such cases resolution by electromyography would be extremely difficult.

## Summary.

In decerebrate cats stretch reflexes from the ankle extensors have been studied in 100 fibres from dissected ventral root filaments. These reflexes have been maximally facilitated by post-tetanic potentiation, as described by GRANIT (1956 a).

In the post-tetanic or potentiated state the reflexes to a constant 10 mm stretch have been found to fall into two main categories: (i) tonic reflexes which go on firing for a long duration in maintained stretch and (ii) phasic ones which only fire one or two spikes on the rising phase of stretch, in spite of any amount of post-tetanic potentiation.

Analysis of spike size showed that the tonic ventral horn cells tended to fire smaller spikes than the phasic ones. From this it was concluded that the tonic motoneurones tend to group themselves among the smaller ventral horn cells.

The difference between tonic and phasic ventral horn cells was maintained when the extensor tonus was raised by decortication of the anterior lobe of the cerebellum.

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# Free Fatty Acid Content of Rat Thoracic Duct Lymph during Fat Absorption.

By

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After absorption from the small intestine long chain fatty acids fed as esters or free acids are found in the thoracic duct lymph mainly in the ester form (cf. BERGSTRÖM and BORGSTRÖM 1955). The free fatty acid content of thoracic duct lymph has been reported to be low or insignificant (BORGSTRÖM 1952 a, RELLER, BENEDICT, MATTSON and BECK 1954).

The significance and origin of the free fatty acid fraction of thoracic duct lymph during fat absorption is not clear. The free fatty acids may be original constituents of the lymph or may have been formed by the action of lipases (clearing factor) *in vivo* or *in vitro* during the collection of the lymph.

With the intention to get further information on the origin of the free fatty acids of thoracic duct lymph during fat absorption we have fed olive oil containing some different [carboxy-$^{14}$C]-labelled fatty acids to rats. The specific activity pattern of the different fatty acid fractions of the lymph fat has been studied.

## Experimental.

The labelled acids used in this investigation were:

I) [1-$^{14}$C] decanoic acid with a specific activity of 122,000 cpm per mg.

II) [1-$^{14}$C] tetradecanoic acid (specific activity 55,000 cpm per mg).

**Table 1.**

*Percentage activity absorbed and recovered in the thoracic duct lymph.*

| Fat fed | % Activity absorbed | % Activity in lymph fat of absorbed activity |
|---|---|---|
| I<br>600 mg 1.9 % $^{14}$C-decanoic acid in olive oil. | 95.2 | 16.2 |
| II<br>940 mg 5 % $^{14}$C-tetradecanoic acid in olive oil. | 97.7 | 41.3 |
| III<br>940 mg 0.9 % $^{14}$C-hexadecanoic acid in olive oil. | 83.6<br>93.3<br>[1]93.1 | 57.0<br>61.0<br>83.1 |
| IV<br>810 mg 4.4 % $^{14}$C-oleic acid in olive oil. | 87.3 | 55.2 |
| V<br>940 mg 3.6 % $^{14}$C-2-methyloctadecanoic acid in olive oil. | 95.7 | 63.2 |
| VI<br>940 mg 3.2 % $^{14}$C-2,2-dimethylnonadecanoic acid in olive oil. | [1]74.1<br>92.5<br>[1]76.5 | 39.5<br>47.4<br>52.3 |

[1] These rats got additional unlabelled olive oil.

III) [1-$^{14}$C] hexadecanoic acid (specific activity 500,000 and 36,000 cpm per mg).
IV) [1-$^{14}$C] oleic acid (specific activity 34,000 cpm per mg).
V) 2,2-dimethyl[1-$^{14}$C]nonadecanoic acid (specific activity 125,000 cpm per mg).

The branched chain fatty acid was synthesised by TRYDING and WESTÖÖ (1955). The oleic acid was prepared according to BERGSTRÖM, PÄÄBO and ROTTENBERG (1952). The saturated straight chain fatty acids were synthesised according to standard procedures.[1]

The following stock solutions of the labelled acids in olive oil were prepared:

I) Decanoic acid (specific activity 2,400 cpm per mg).
II) Tetradecanoic acid (s. a. 2,750 cpm per mg).
III) Hexadecanoic acid (s. a. 4,500 cpm per mg).
IV) Oleic acid (s. a. 1,500 cpm per mg).
V) 2,2-Dimethylnonadecanoic acid (s. a. 4,000 cpm per mg).

[1] These acids were kindly supplied by Professor S. BERGSTRÖM.

**Table 2.**

*Weight and distribution of activity of different lymph fat fractions.*

| Fat fed | S. A. | Neutral fat | | Phospholipids | | Free fatty acids | |
|---|---|---|---|---|---|---|---|
| | | weight mg | s. a. | weight mg | s. a. | weight mg | s. a. |
| I..... | 100 | 522 | 18 | 31.0 | 6 | 36.4 | 17 |
| II..... | 100 | 501 | 76 | 46.0 | 23 | 64.9 | 47 |
| III..... | 100 | 779 | 50 | — | — | 50.2 | 20 |
| | 100 | 669 | 64 | 24.8 | 25 | 60.2 | 38 |
| | 100 | 1,422 | 43 | — | — | 114.5 | 30 |
| IV..... | 100 | 686 | 46 | 49.1 | 35 | 55.0 | 17 |
| V..... | 100 | 690 | 70 | — | — | 48.4 | 25 |
| VI..... | 100 | 1,444 | 13 | — | — | 83.4 | 37 |
| | 100 | 1,148 | 30 | 52.5 | 14 | 91.9 | 73 |
| | 100 | 977 | 20 | — | — | 71.5 | 42 |
| VII | | | | | | | |
| Unlabelled | 100 | 1,085 | 0 | — | — | 87.8 | 14 |
| olive oil[1] | 100 | 2,580 | 0 | — | — | 101.0 | 10 |

[1] $^{14}C$-labelled palmitic acid added to the lymph in amounts of 12.26 mg and 11.95 mg with a specific activity of 5,400 cpm and mg = 100 in the table.

The radioactivities were determined with a windowless GM-tube (background effect 40 cpm), after plating 1 mg of the substances on aluminium planchets. The decanoic acid was plated as the sodium salt.

Adult, male rats weighing about 250 g were used. The cannulation of the thoracic duct and the post-operative treatment have been described by BERGSTRÖM, BORGSTRÖM and BLOMSTRAND.

About 24 hours after the operation 1 ml of the stock solutions were fed by stomach tube. The lymph from the next 24 hours (80—130 ml) was collected in 150 ml ethanol to prevent any enzymatic hydrolysis during the collection time. In the isotope dilution experiments unlabelled olive oil was fed and 10—12 mg of labelled hexadecanoic acid were dissolved in the ethanol before the lymph collection started.

The lymph was refluxed with 20 volumes ethanol-ether (3 : 1 v, v) for 1 hour. The filtrate was evaporated *in vacuo* and the residue (2—4 ml) extracted five times with 50 ml portions of light petroleum (b. p. 40—60)—chloroform (2 : 1 v, v). Less than 1 per cent of the activity remained in the water phase after the extraction. No acidification was necessary for the extraction of the free fatty acids. The phospholipids and lymph neutral fat including free fatty acids were separated on columns of silicic acid (BORGSTRÖM 1952 a). The neutral lipids and

the free fatty acids were separated on Amberlite IRA-400 (BORGSTRÖM 1952 b). In the experiments in which decanoic acid was fed the free acid fraction was extracted with an alkaline solution of ethanol-water 1 : 1 from the light petroleum solution of the chloroform eluate. The weight of the fatty acids of the different fractions after saponification was determined after drying over paraffine and phosphorous pentoxide. In addition the free fatty acid fraction was titrated with 0.05-n NaOH (bromthymol blue). The faeces and the urine were collected separately during the next two days. The faeces were digested in 30 per cent KOH on a steam bath for 12 hours and the fatty acids extracted with ether after acidification. The amount of activity absorbed was calculated as the difference between fed activity and faecal activity.

## Results and Discussion.

The figures for free fatty acid content of rat thoracic duct lymph during fat absorption have been obtained after isolation of the free fatty acid fraction with the ion-exchanger resin Amberlite IRA-400 (except for the decanoic acid), by weight, titration and in some cases by isotope dilution procedure. All three methods gave figures indicating a free fatty acid content of 7.2 (3.8—11.8) per cent calculated on lymph neutral fat (glycerides, sterols and sterol esters) or 2.2 (0.86—3.61) meq/l lymph (Table 3).

The figures are higher than those reported by RELLER et al. who found free fatty acid values of the acetone soluble lipids of rat lymph ranging from 0 to 2.7 per cent with a mean value of 1.1 per cent. The acetone soluble lipids of RELLER et al. correspond to neutral lipids + free fatty acids. Our figures are lower than those recently reported for rats by YOUNG and FREEMAN. These authors reported the content of free fatty acids in thoracic duct lymph to be 15.7 (8.8—30.1) per cent calculated on triglycerides. The figures are not quite comparable as in our experiments the lymph was collected over a 24 hour period and thus also includes lymph from fasting periods while in the experiments of YOUNG and FREEMAN, the lymph was collected during short periods during active fat absorption.

A study of the specific activities of the free fatty acid fractions when different, labelled acids have been fed, further shows differences which are not parallel to those of the neutral fat fractions. When labelled straight chain or a mono-methyl branched acid (TRYDING and WESTÖÖ 1956) were fed, the specific activity of the free fatty acids isolated was lower than that of the neutral fat fatty acids. After feeding 2,2-dimetylnonadecanoic acid,

**Table 3.**

*Amounts and activity of free fatty acids of thoracic duct lymph.*

| Fat fed | Free fatty acids obtained by: | | | Free fatty acids in per cent of neutral fat (titr.) | S. a. of free fatty acids in % of s. a. of neutral fat fatty acids | Free fatty acids meq per l lymph |
|---|---|---|---|---|---|---|
| | weight | titration | isotope dilution | | | |
| I ... | 36.4 | 31.7 | — | 6.1 | 95 | 1.06 |
| II ... | 64.9 | 59.0 | — | 11.8 | 61 | 2.62 |
| III ... | 50.2 | 56.6 | — | 7.3 | 40 | 2.23 |
| | 60.2 | 49.7 | — | 7.4 | 59 | 1.35 |
| | 114.5 | 116.1 | — | 8.2 | 70 | 3.61 |
| IV ... | 55.0 | 60.2 | — | 8.8 | 31 | 2.13 |
| V ... | 48.4 | 32.0 | — | 4.6 | 36 | 0.86 |
| VI ... | 83.4 | 54.8 | — | 3.8 | 285 | 1.66 |
| | 91.9 | 97.5 | — | 8.5 | 243 | 3.01 |
| | 71.5 | 65.8 | — | 6.7 | 208 | 2.12 |
| VII ... | 87.0 | 88.6 | 88.8 | 8.2 | — | 3.14 |
| | 101.0 | 110.1 | 120.0 | 4.3 | — | — |
| | | | | Mean 7.2 | | 2.20 |

however, the specific activity of the free fatty acids was 200—300 per cent of that of the neutral fat fatty acids. This indicates that the free fatty acids cannot have been formed by lipolysis of the neutral fat (nor the phospholipids). None of these fractions are, however, homogeneous; the neutral fat fraction containing triglycerides and cholesterolesters and the phospholipid fraction containing different species. But the free fatty acid fraction is so large from a quantitative point of view that it is clear that the only fraction that on lipolysis could have been the main origin of the free fatty acids is the triglyceride fraction. It therefore seems most probable from the isotope data that the free fatty acids of the lymph are mainly original constituents of the lymph. The results recently obtained by YOUNG and FREEMAN also speak in favour of the above interpretation. When trielaidin containing fat was fed to rats the concentration of the elaidic acid was much lower in the free fatty acid fraction than in the glyceride fraction. After injecting heparin intravenously, when an up to fivefold increase in the concentration of the thoracic duct free fatty acids

was found, the elaidic acid in the free fatty acid fraction, however, increased indicating hydrolysis of lymph glycerides *in vivo*.

As the quantitative composition of the lymph fat in our experiment is the same irrespectively of the labelled acid fed, there are also differences in the partition of the different labelled acids between the different lymph fractions. In the case of the 2,2-dimethylnonadecanoic acid about 14 per cent of the total lymph activity is found in the free fatty fraction, the corresponding figure for the other acids being about 6 per cent. As the amount of fed labelled acids is small in these experiments the concentration of labelled acids in the free fatty acid fraction is low, for the 2,2-dimethylnonadecanoic acid only about 1 per cent.

The reason for the different metabolic pattern of the 2,2-dimethylnonadecanoic acid most probably has to be found on the enzymatic level. As a further indication of this it was earlier found (BERGSTRÖM, BORGSTRÖM, TRYDING and WESTÖÖ) that glyceride ester bonds with 2,2-dimethyloctadecanoic acid could not be split by pancreatic lipase at a rate corresponding to that of straight chain fatty acids. The incomplete incorporation into ester bonds of the 2,2-dimethyloctadecanoic acid has been demonstrated also *in vivo* (BLOMSTRAND, TRYDING and WESTÖÖ). The results obtained with the decanoic acid are at variance with those given by BLOMSTRAND, who reported that all of the decanoic acid transported via the lymph after absorption was in the ester form. In our experiment we found 4.1 per cent of the labelled lymph decanoic acid in the form of the free acid. This difference in results has to be found in the different methods used for the isolation of the free fatty acid fraction.

## Summary.

Free fatty acid has been found to make up a significant part of rat thoracic duct lymph lipids during fat absorption.

The results obtained indicate that the free fatty acids of thoracic duct lymph are original constituents of the lymph rather than derived by hydrolytic processes in the lymph.

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# Some Aspects of the Basal Tone of the Blood Vessels.

By

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Even after the cutting of all vasomotor nerves, the blood vessels in some areas, *e. g.* in the skeletal muscles, still exhibit a remarkably pronounced tone, while under the same circumstances the vessels of certain skin areas, dominated by an abundance of arteriovenous anastomoses, are almost maximally dilated (for lit. see FOLKOW 1955). It is widely believed that such a 'basal vascular tone' is a consequence of specific constrictor agents in the blood stream (see *e. g.* LE COMPTE 1941). Thus it has been suggested that such agents are released from the spleen, exerting a tonical influence on the blood vessels (GRANAAT 1953), and it seems to be widely assumed that the secretion from the suprarenal medulla makes a considerable contribution to the maintenance of the basal vascular tone. It is quite clear that any release of vasoconstrictor agents into the blood, if only reaching significant concentrations, will influence vascular tone, but from this circumstance it in no way follows that basal vascular tone is only or mainly a consequence of blood-born constrictor agents. It may equally well be due to a local mechanism, such as an inherent automaticity of the vascular smooth muscle cells. — The present study is an attempt to analyse which of the suggested mechanisms are of dominant importance for the maintenance of a basal vascular tone.

## Method.

Experiments have been performed on 20 cats, anaesthetized with chloralose, 50—70 mg per kg of body-weight. In a first series the level of the basal tone and the reactions to biogenic constrictor agents of the vessels of the limb muscles and the skin of the paw were studied. In order to measure the cutaneous blood flow of the right paw the big saphenal vein was dissected free at the ankle joint, while other superficial veins were ligated. After heparinization the outflow from the saphenal vein was measured by an optical drop recorder operating an ordinate writer. The blood flow from the muscles of the lower part of the skinned left hind limb was measured by a similar blood flow recorder unit connected to the femoral vein at the level of the knee joint, while the blood flow of the left paw was completely obstructed by a tight ligature at the ankle joint. In such a preparation the femoral vein drains only bone and muscle tissue. The left limb was protected from drying and cooling by gauze moistened with warm Tyrode solution and a thin rubber cover. In some of the experiments the skin cover over the muscles was left intact, which did not significantly influence the estimated level of vascular tone in the left limb as long as the circulation of the left paw was obstructed. The outflowing blood was returned to the animal by a continuous intravenous drop infusion, kept in step with the outflow. The two abdominal sympathetic chains were extirpated from L 3 down to the sacral region, and the femoral, saphenal and sciatic nerves were cut in both limbs in order to obtain a complete nervous 'decentralization' of their blood vessels. The intestines with the exception of the first part of the jejunum were extirpated and a small cannula was inserted into the central end of the inferior mesenteric artery to allow intra-arterial injections and infusions to the two hind limbs. The blood pressure was measured with a mercury manometer connected to the caudal extension of the abdominal aorta and the inflow pressure to the two limbs could be kept constant for a given period, if desired, by a screw clamp on the abdominal aorta proximally to the point of pressure measurement.

In a second series of the experiments the postulated release of vasotonic agents from the spleen was studied. Beside the above-mentioned vascular regions the reactions of the vessels of the jejunum were sometimes also recorded, to be used as an additional indicator of a possible release of constrictor agents from the spleen. All nerve fibres running around the superior mesenteric artery were then cut and in addition the splanchnic nerves were sectioned. The activity of the sensitized nictitating membrane, denervated 10—14 days earlier, was also recorded. — The splenic nerves were carefully dissected free and prepared for stimulation, the splenic artery was cut and reconnected again by a rubber catheter to allow close arterial injections into the spleen. A cannula was inserted into the central end of a branch of the superior mesenteric vein to make possible a comparison of the effects on the above-mentioned indicators of intraportal and intrasplenic injections of equal amounts of catechol amines.

In some of these experiments the right suprarenal gland was extirpated and the left one denervated right from the beginning, to eliminate the resting secretion of catechol amines; in others this procedure was performed during the course of the experiment.

## Results.

Confirming earlier observations it was easily demonstrated that the basal tone of the vessels of the skeletal muscle is considerable, as the regional peripheral resistance in most cases can be decreased 5—7 times by close arterial injection of supramaximal amounts of acetylcholine or adenosine triphosphate. This tone is not a matter of a catechol amine secretion from the suprarenal glands, as extirpation or denervation of these glands did not decrease the resistance to the blood flow. As regards the skin vessels of the paw, the regional peripheral resistance can here only be reduced some 20 to 50 per cent even by huge amounts of vasodilator agents, indicating that this vascular area, dominated by an abundance of arteriovenous anastomoses subserving the central control of heat loss, are almost maximally dilated when deprived of their vasoconstrictor nerves, as long as the discharge from the suprarenal medulla is at a basal level or quite eliminated by denervation of the suprarenal glands. The lack of further dilation of the paw vessels can hardly be due to a low sensitivity to the mentioned dilator agents as a pronounced dilation was obtained even by minor amounts if only the initial vascular tone was kept at a high level, *e. g.* by sympathetic stimulation or infusion of noradrenaline.

On the other hand, when noradrenaline, adrenaline, serotonin, angiotonin or vasopressin — all known to be produced in the body and under at least some circumstances present in the blood stream — were infused or injected intra-arterially to the hind limbs, the vasoconstrictor effects were, with only a few occasional exceptions for vasopressin, quite regularly more intense in the skin than in the muscles at all concentrations used, which should be contrasted to the fact that basal vascular tone is considerably more pronounced in the muscles than in the skin of the paw (fig. 1).

As regards the question of a possible release of vasotonic agents from the spleen, it was observed that even intense stimulation of the splenic nerves, which caused maximal constrictor responses of the spleen itself, had no secondary effects whatsoever on the

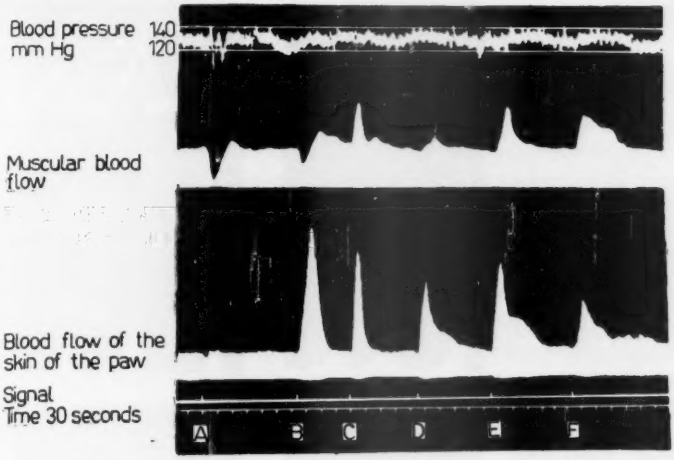


Fig. 1. Blood pressure, muscular blood flow in the left hind limb and cutaneous blood flow in the paw of the right hind limb of the cat.
Intra-arterial injection of:
A: 2 μg Acetylcholine,
B: 1 μg Adrenaline,
C: 1 μg Noradrenaline,
D: Serotonin,
E: Vasopressin,
F: Angiotonin.
As the samples of serotonin, vasopressin and angiotonin were not quite pure, the *absolute* dosage is not given.

tone of either the vessels of the skin, the muscles or the intestine, in spite of a high sensitivity of these vascular regions to even minute amounts of biogenic constrictor agents. Only on stimulation of the splenic nerves at rates that can be considered to be supramaximal could a slight contraction of the highly sensitive denervated nictitating membrane sometimes be observed. This effect, however, seems to be simply a matter of an 'overflow' of excess amounts of the liberated constrictor fibre transmitter, known to occur under such circumstances (see CELANDER 1954), rather than to be due to a physiological release to the blood stream of a specific vasotonic agent. Further, when different concentrations of adrenaline and noradrenaline were injected intra-arterially to the spleen there was never any evidence of an additional release of a splenic vasotonic agent into the blood stream, as injections of the same amounts of the catechol amines directly into the portal vein always induced considerably stronger response of the indicators (fig. 2), which were still more intense

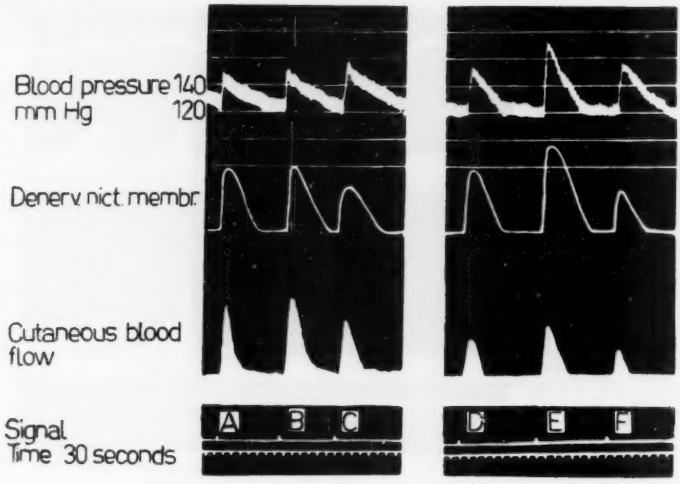


Fig. 2. Blood pressure, contraction of the chronically denervated nictitating membrane and cutaneous blood flow in the paw of the right hind limb of the cat.
A: Intravenous injection of 2 $\mu$g Adrenaline,
B: Injection into the portal vein of 10 $\mu$g Adrenaline,
C: Injection into the splenic artery of 10 $\mu$g Adrenaline,
D: Intravenous injection of 2 $\mu$g Noradrenaline,
E: Injection into the portal vein of 10 $\mu$g Noradrenaline,
F: Injection into the splenic artery of 10 $\mu$g Noradrenaline.

when the liver too was bypassed by injection of the catechol amines into the brachial vein.

## Discussion.

The present experiments indicate that the vessels of the skin of the paw are more sensitive than the vessels of the skeletal muscles to a series of biogenic vasoconstrictor agents that at least under some circumstances are known to be released into the blood stream. As the tone of the acutely denervated vessels of the skeletal muscles is nevertheless considerable while that of the vessels of the skin of the paw is almost negligible it is therefore unlikely that basal vascular tone on the whole is a consequence of blood-born vaso-active agents. The difference in basal vascular tone in the two areas is, however, readily explained as a result of an automaticity of the vascular smooth muscle cells. A number of direct studies of the behavior of minute blood vessels

and isolated arteries present strong evidence for the existence of such an automaticity (for lit. see *e. g.* FOLKOW 1953), and it is also in accordance with the characteristics of other types of smooth muscles (see BOZLER 1948). It is obvious that a rhythmic, unsynchronized activity of vascular smooth muscle cells must create a vascular tone, the more pronounced the stronger this myogenic activity is. Automaticity is probably to be looked upon as a basic characteristic both of the heart and of most types of smooth muscle, though it seems to be more or less completely lost in certain types of highly specialized smooth muscle cells which to allow an exact performance are quite dominated by a central nervous regulation, *e. g.* the intrinsic smooth muscles of the eye. In a sense the cutaneous arteriovenous anastomoses show a similar type of specialization with a complete central nervous domination to allow a sensitive adjustment of the heat loss from the body. Such a mechanism, subserving the organism as a whole, would have been much interfered with if the tone of these specialized vessels had also been influenced by a pronounced myogenic activity. A loss of automaticity will, however, have the consequence that after sympathectomy the vessels will become almost maximally dilated provided the blood does not contain significant amounts of constrictor agents. The fact that the vessels of the paw do become almost completely dilated on constrictor fibre section, indicates that they show little myogenic activity and that only very small amounts of constrictor substances are present in the blood stream under 'resting' physiological conditions. These vessels are so sensitive to minute amounts of *e. g.* catechol amines that even a small blood concentration would be revealed by a definite constrictor response. The observations of LE COMPTE (1941) are in fact in agreement with the present study as far as cutaneous vessels are concerned but it must be stressed that these vessels can not be looked upon as representative of all vascular regions if one wants to analyse the background of basal vascular tone, as has mostly been the case in older studies. No evidence of a sympathetically controlled release of specific vasotonic factors from the spleen could be obtained, even though very sensitive indicators within the vascular system were utilized which react promptly to even minute amounts of vasoconstrictor substances. The present experiments are therefore not able to confirm GRANAAT's hypothesis, which was based on an analysis of the differences in the

blood pressure responses that were induced by intrasplenic and intraportal injections of catechol amines or by splenic nerve stimulation. It should be stressed, however, that for such an analysis the arterial blood pressure alone is no secure indicator of changes in vascular tone. When the spleen is brought to constriction the arterial blood pressure may also be markedly influenced by increases in circulating blood volume with consequent effects on cardiac output, by viscosity changes due to an increased hematocrit not to mention the effects of an increased peripheral resistance within the spleen itself. Had the differences in the blood pressure responses in GRANAAT's study really been due to a release of a specific vasoconstrictor agent from the spleen it would be expected that at least in some of the present experiments the responses of the different sensitive indicators would have revealed such a release. This was not the case, however, and therefore the hypothesis that the spleen releases a vasotonic agent could not be confirmed. Whether the heart may be influenced by some factor released from the spleen, as REIN (1949, 1951) suggested, is beyond the scope of the present investigation.

## Summary.

In experiments on cats the nature of the basal vascular tone that remains after sympathectomy has been analysed.

The basal tone of the vessels of the skeletal muscles is quite marked while the vessels of the paw, with its abundance of arteriovenous anastomoses, become almost maximally dilated after constrictor fibre section. On the other hand, the latter type of vessels are more sensitive to known biogenic constrictor substances. No evidence of a release of vasotonic agents from the spleen could be obtained.

It is therefore suggested that basal vascular tone can hardly be a consequence of circulating constrictor agents, but is probably a matter of smooth muscle automaticity, more or less pronounced in different regions depending on the degree of functional differentiation of the vessels.

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# Variations in the Total Amounts of Polyenoic Fatty Acids in Chicks and Rats Reared on a Nearly Fat-free Diet.

By

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Previous studies from this laboratory have shown that when chicks are reared on "fat-free" diets the amount of trienoic fatty acid in the organs studied is higher than when the diet contains a certain level of peanut oil (DAM, KRISTENSEN, NIELSEN, PRANGE and SØNDERGAARD 1956). Other studies from our laboratories show that the same holds for rats (ENGEL, AAES-JØRGENSEN, FUNCH and DAM, yet unpublished). Certain observations from other laboratories point in the same direction (*e. g.*, RIECKEHOFF, HOLMAN and BURR 1949, REISER 1951).

It was therefore thought to be of interest to find out how the total amount of trienoic acid compared with the amounts of the other polyenoic acids varies in the experimental animals on the nearly fat-free dietary regimen.

Two series of experiments were carried out, viz. one with chicks and one with rats.

### Chick Experiment.

H. DAM and G. KOFOED NIELSEN.

## Experimental.

Two groups of day-old chicks (nine chicks in each group) were given a "normal" diet (DAM and SØNDERGAARD 1953). When they were 14 days old, the chicks in one of the groups were killed with chloroform, while those in the other group were shifted to the "fat-free" diet no. 3, indicated in table 1. The chicks in this latter group were killed with chloroform at the age of 42 days, that is after 4 weeks on the "fat-free" diet.

**Table 1.**

*Diet no. 3 (for chicks).*

| | | |
|---|---|---|
| Casein, crude[1] | 20 | g |
| Gelatin | 8 | g |
| Salt mixture no. 4[2] | 5.17 | g |
| Vitamin mixture no. 4[3] | 1 | g |
| d,l-α-tocopherol acetate[4] | | 10 mg |
| Vitamin K substitute[5] | | 10 mg |
| Choline chloride | 0.15 | g |
| Sucrose | 66.58 | g |

The chicks received vitamins A and $D_3$ in filtrol treated peanut oil, one drop twice a week, furnishing each animal with 220 I. U. A and 20 I. U. $D_3$ per day. The A and D solution consisted of 1.7 g vitamin A concentrate, natural ester form, containing 838,000 I. U. A per g, 0.63 g vitamin $D_3$ concentrate containing 200,000 I. U. per g and 47.67 g filtrol treated peanut oil; total 50 g. The vitamin A concentrate was kindly furnished by Distillation Products Industries, Rochester, N. Y.; the vitamin $D_3$ concentrate was kindly furnished by Ferrosan Ltd., Copenhagen.

[1] "Dairinex", from A/S Dansk Mejeri Industri & Export Kompagni, Stege, Denmark, containing 1.2 % fat. The fat in the crude casein contained 1.7 % dienoic, 0.7 % trienoic, and 0.1 % tetraenoic acid (cf. DAM, KRISTENSEN, NIELSEN, PRANGE and SØNDERGAARD 1956). The crude casein and the A,D-solution will furnish each chick with about 1.8 mg dienoic, 0.17 mg trienoic and 0.24 mg tetraenoic acid per day. In the whole 4-week period this will amount to about 50 mg dienoic, 4.8 mg trienoic and 6.7 mg tetraenoic.

[2] Consisting of: 2,800 g secondary calcium phosphate, 875 g calcium carbonate, 404 g desiccated magnesium sulfate, Ph. Dan., 460 g potassium chloride, Ph. Dan., 500 g sodium chloride, Ph. Dan., 100 g ferric citrate (about 17.5 % Fe), 23 g manganese sulfate (water-free), 2 g cupric sulfate, 5 aq., Ph. Dan., 1 g zinc sulfate, 7 aq., Ph. Dan., 1 g aluminium sulfate, 18 aq., Ph. Dan., 1 g magnesium silicate, Ph. Dan., 1 g diiodotyrosine, Ph. Dan., 0.05 g cobalt carbonate; total 5,168.05 g.

[3] Consisting of: 3 mg thiamine hydrochloride, 4 mg riboflavin, 50 mg nicotinic acid, 12 mg calcium pantothenate, 3.5 mg pyridoxine hydrochloride, 0.1 mg biotin, 2 mg folic acid, 925.4 mg sucrose; total 1,000 mg.

[4] Ephynal, "Roche", kindly furnished by the manufacturer.

[5] Dicalcium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid ester (Synkavit, "Roche"), kindly furnished by the manufacturer.

The crop of the chicks was empty when the animals were killed. After being cut into pieces each chick was treated on steam bath with occasional stirring for $2^1/_2$ to 3 hours with a mixture of 2 ml 30 % KOH (w/v) and 0.8 ml 96 % (w/w) ethanol per g body weight. The resulting homogeneous solution was decanted from undissolved parts of the skeleton and diluted to one liter with water-ethanol (5 : 2 by vol.); 100 ml of the solution was acidified with concentrated hydrochloric acid (0.12 ml per g of the total body weight) and shaken out three times with petroleum ether. The undissolved bones were dissolved in 25 ml concentrated hydrochloric acid and likewise shaken out three times with petroleum ether. One tenth of this petroleum ether solution was added to the corresponding part of the main portion. The amount of fatty acids obtained by the hydrochloric acid treatment of the bones was about 1 % of the total fatty acids. The combined petroleum ether solution representing one tenth of the fatty acids in the chick was dried with sodium sulfate, evaporated *in vacuo*, weighed and alkali-isomerized as described by HAMMOND and LUNDBERG (1953).

## Results and Discussion.

The results are presented in tables 2 a and b.

Comparison between the data found for the chicks killed after 2 weeks on the "normal diet" and for those having received the "normal diet" for 2 weeks and thereafter the "fat-free" diet for 4 weeks shows the following:

During the four-week "fat-free" regimen the body weight and the total amount of fatty acids in the chicks has increased. The amount of total fatty acids per g chick is nearly unchanged. The absolute amounts of the individual polyenoic fatty acids in the body exhibit a decrease in dienoic of 404 mg, *i. e.* to about half of the original value. Trienoic has increased 89 mg, *i. e.* to almost three times the original value. Tetraenoic has increased 37 mg, *i. e.* to about one and a half of the original value, whereas penta- and hexaenoic have remained nearly constant.

From the figures it is concluded that the increase in trienoic must have come from dienoic. The lesser increase in tetraenoic must have had the same origin. The small amount of polyenoic acids contained in the diet (cf. p. 143) does not influence these conclusions.

The percentage of the individual polyenoic acids calculated on the basis of total fatty acids shows likewise a considerable decrease in dienoic, but only a slight increase in trienoic, and a decrease in tetra-, penta- and hexaenoic. When calculated per g body weight the most appalling change is a drastic reduction in dienoic.

**Table 2a.**

*Polyenoic fatty acids in 14 days old chicks reared on "normal diet".*

| Chick no. | | 4211 | 4212 | 4214 | 4215 | 4217 | 4221 | 4224 | 4225 | 4227 | Average |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Weight in g when killed | | 101 | 100 | 92 | 87 | 92 | 82 | 89 | 83 | 72 | 89 |
| Total amount of fatty acids, g[1] | | 6.48 | 6.67 | 6.41 | 5.24 | 7.54 | 4.36 | 5.62 | 5.06 | 5.56 | 5.88 |
| mg fatty acids/g chick | | 64 | 67 | 70 | 60 | 82 | 53 | 63 | 61 | 77 | 66 |
| mg in total body | dienoic | 1,117 | 1,110 | 844 | 661 | 890 | 665 | 836 | 556 | 582 | 807 |
| | trienoic | 56 | 51 | 37 | 50 | 61 | 36 | 62 | 44 | 38 | 48 |
| | tetraenoic | 87 | 117 | 74 | 84 | 88 | 83 | 56 | 60 | 52 | 78 |
| | pentaenoic | 70 | 66 | 42 | 16 | 23 | 17 | 38 | 29 | 29 | 37 |
| | hexaenoic | 80 | 68 | 39 | 41 | 41 | 38 | 56 | 43 | 46 | 50 |
| | polyenoic | 1,410 | 1,412 | 1,036 | 852 | 1,103 | 839 | 1,048 | 732 | 747 | 1,020 |
| per cent of total fatty acids[1] | dienoic | 17.25 | 16.47 | 13.14 | 12.61 | 11.81 | 15.24 | 14.88 | 10.99 | 10.47 | 13.65 |
| | trienoic | 0.87 | 0.76 | 0.58 | 0.96 | 0.81 | 0.83 | 1.10 | 0.87 | 0.68 | 0.83 |
| | tetraenoic | 1.34 | 1.75 | 1.15 | 1.61 | 1.16 | 1.89 | 1.00 | 1.19 | 0.94 | 1.34 |
| | pentaenoic | 1.08 | 0.99 | 0.66 | 0.31 | 0.30 | 0.39 | 0.68 | 0.57 | 0.53 | 0.61 |
| | hexaenoic | 1.23 | 1.02 | 0.61 | 0.78 | 0.55 | 0.88 | 0.99 | 0.85 | 0.82 | 0.86 |
| mg/g chick | dienoic | 11.1 | 11.1 | 9.2 | 7.6 | 9.7 | 8.1 | 9.4 | 6.7 | 8.1 | 9.0 |
| | trienoic | 0.6 | 0.5 | 0.4 | 0.6 | 0.7 | 0.4 | 0.7 | 0.5 | 0.5 | 0.5 |
| | tetraenoic | 0.9 | 1.2 | 0.8 | 1.0 | 1.0 | 1.0 | 0.6 | 0.7 | 0.7 | 0.9 |
| | pentaenoic | 0.7 | 0.7 | 0.5 | 0.2 | 0.3 | 0.2 | 0.4 | 0.3 | 0.4 | 0.4 |
| | hexaenoic | 0.8 | 0.7 | 0.4 | 0.5 | 0.4 | 0.5 | 0.6 | 0.5 | 0.6 | 0.6 |

[1] Including the non-saponifiable matter, the amount of which is considered negligible for the present purpose.

**Table 2b.**

*Polyenoic fatty acids in 42 days old chicks reared on "normal diet" for the first 2 weeks, thereafter on "fat-free diet no. 3" for 4 weeks.*

| Chick no. | | 4210 | 4213 | 4216 | 4219 | 4220 | 4222 | 4223 | 4226 | 4228 | Average |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Weight in g when killed | | 193 | 330 | 242 | 248 | 220 | 343 | 208 | 166 | 214 | 246 |
| Total amount of fatty acids g[1] | | 11.9 | 21.7 | 14.9 | 12.2 | 14.2 | 20.0 | 15.7 | 8.0 | 12.2 | 14.5 |
| mg fatty acids/g chick | | 62 | 66 | 62 | 49 | 65 | 52 | 75 | 48 | 57 | 59 |
| mg in total body | dienoic | 411 | 525 | 408 | 411 | 367 | 484 | 372 | 302 | 351 | 403 |
| | trienoic | 127 | 264 | 167 | 104 | 114 | 142 | 104 | 91 | 124 | 137 |
| | tetraenoic | 126 | 139 | 137 | 106 | 91 | 104 | 96 | 102 | 138 | 115 |
| | pentaenoic | 32 | 43 | 40 | 40 | 30 | 38 | 27 | 30 | 32 | 35 |
| | hexaenoic | 52 | 89 | 46 | 46 | 38 | 48 | 22 | 38 | 50 | 48 |
| | polyenoic | 748 | 1,060 | 798 | 707 | 640 | 816 | 621 | 563 | 695 | 738 |
| per cent of total fatty acids[1] | dienoic | 3.46 | 2.42 | 2,74 | 3.37 | 3.29 | 2.42 | 2.37 | 3.77 | 2.88 | 2.97 |
| | trienoic | 1.07 | 1.22 | 1.12 | 0.85 | 0.80 | 0.71 | 0.66 | 1.14 | 1.02 | 0.95 |
| | tetraenoic | 1.06 | 0.64 | 0.92 | 0.95 | 0.64 | 0.52 | 0.61 | 1.27 | 1.13 | 0.86 |
| | pentaenoic | 0.27 | 0.20 | 0.27 | 0.33 | 0.21 | 0.19 | 0.17 | 0.37 | 0.26 | 0.25 |
| | hexaenoic | 0.44 | 0.41 | 0.31 | 0.38 | 0.27 | 0.24 | 0.14 | 0.48 | 0.41 | 0.34 |
| mg/g chick | dienoic | 2.1 | 1.6 | 1.7 | 1.7 | 1.7 | 1.4 | 1.8 | 1.8 | 1.6 | 1.7 |
| | trienoic | 0.7 | 0.8 | 0.7 | 0.4 | 0.5 | 0.4 | 0.5 | 0.5 | 0.6 | 0.6 |
| | tetraenoic | 0.7 | 0.4 | 0.6 | 0.5 | 0.4 | 0.3 | 0.5 | 0.6 | 0.6 | 0.5 |
| | pentaenoic | 0.2 | 0.1 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 |
| | hexaenoic | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 |

[1] Including the non-saponifiable matter, the amount of which is considered negligible for the present purpose.

### Rat Experiment.

H. DAM and P. F. ENGEL.

## Experimental.

Four newly weaned rats weighing 36 to 40 g each were killed and treated by the same method as the chicks with the exception that the alimentary canal was removed, and that the dilution of the alkaline solution was omitted, so that the total amount of fatty acids from each rat was isolated and used for the analyses.

Six other newly weaned rats of the same weights and from the same litters were reared for 18 weeks on the fat-free diet no. 181, indicated in table 3. At the end of this period the six rats were killed and, after removal of the alimentary canal, treated as in the chicken experiment.

**Table 3.**

*Diet no. 181 (for rats).*

| | |
|---|---|
| Casein, vitamin test[1] | 20 g |
| Salt mixture no. 1[2] | 5 g |
| Vitamin mixture no. 1[3] | 0.5 g |
| Choline chloride | 0.5 g |
| Sucrose | 74 g |

The animals received vitamins A and $D_3$ in aqueous colloidal solution, 0.1 ml 3 times a week, furnishing each rat with 17 I. U. A and 2.6 I. U. $D_3$ per day. The solution consisted of 0.25 g vitamin A palmitate ($1.6 \times 10^6$ I. U. per g), 0.06 g solution of vitamin $D_3$ in soybean oil ($1.0 \times 10^6$ I. U. per g), 3 g Tween 80, 0.25 g citric acid, 0.03 g Jonol (2,6-ditertiary-butyl-p-cresol as antioxidant), distilled water to make 1 liter.

[1] From Genatosan Ltd., Loughborough, England, containing 0.11 % fat. The vitamin test casein furnishes each rat with about 0.04 mg dienoic, 0.015 mg trienoic and 0.022 mg tetraenoic acid per day. In the whole 18 week period this will amount to about 5 mg dienoic, 1.9 mg trienoic and 2.8 mg tetraenoic acid. The amount of polyenoic acids in the aqueous vitamin A,D-solution is negligible.

[2] McCollum & Simmonds' salt mixture no. 185, supplemented with 0.131 % $CuSO_4$, $5H_2O$, 0.52 % $MnSO_4$, $4H_2O$ and 0.013 % KI.

[3] 0.5 g of the mixture consisted of: 0.05 mg biotin, 0.05 mg folic acid, 5 mg ascorbic acid, 5 mg thiamine hydrochloride, 5 mg riboflavin, 5 mg pyridoxine hydrochloride, 5 mg calcium pantothenate, 8 mg nicotinic acid, 15 mg inositol, 35 mg p-aminobenzoic acid, 1 mg vitamin K substitute (Synkavit, "Roche"), 5 mg dl-α-tocopherol acetate (Ephynal, "Roche") and sucrose to make a total of 500 mg.

## Results and Discussion.

After 18 weeks on the fat-free regimen the rats showed typical skin signs of fat deficiency. The amount of intraperitoneal fat was considerable.

The results of the analyses are presented in table 4.

During the 18 weeks on the fat-free diet the body weight and total amount of fatty acids have increased. The total amount of fatty acids per g carcass has increased slightly.

**Table 4.**

*Polyenoic fatty acids in rats.*

| | | Weanling rats | | | | | Rats reared on fat-free diet no. 181 for 18 weeks from weaning | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rat no. | | 1 | 2 | 3 | 4 | Average | 5 | 6 | 7 | 8 | 9 | 10 | Average |
| Weight of carcass (minus digestive tract), g | | 40.9 | 36.2 | 36.3 | 37.9 | 37.8 | 147 | 183 | 185 | 175 | 197 | 183 | 179 |
| Total amount of fatty acids, g[1] | | 4.90 | 3.20 | 22.70 | 4.75 | 3.89 | 20.60 | 23.50 | 18.98 | 19.08 | 28.20 | 25.80 | 22.74 |
| mg fatty acids[1]/g carcass | | 120 | 89 | 75 | 125 | 102 | 140 | 128 | 103 | 109 | 143 | 141 | 127 |
| mg in carcass | dienoic | 577 | 378 | 304 | 522 | 446 | 119 | 150 | 91 | 95 | 23 | 126 | 101 |
| | trienoic | 49 | 25 | 17 | 48 | 35 | 231 | 315 | 290 | 274 | 313 | 343 | 294 |
| | tetraenoic | 107 | 91 | 76 | 106 | 95 | 58 | 82 | 82 | 86 | 110 | 103 | 87 |
| | pentaenoic | 53 | 53 | 41 | 50 | 49 | 10 | 21 | 19 | 17 | 14 | 28 | 18 |
| | hexaenoic | 65 | 59 | 52 | 67 | 61 | 35 | 63 | 84 | 32 | 56 | 52 | 54 |
| | polyenoic | 851 | 606 | 490 | 793 | 685 | 453 | 631 | 566 | 504 | 516 | 652 | 554 |
| per cent of total fatty acids[1] | dienoic | 11.78 | 11.80 | 11.26 | 10.99 | 11.46 | 0.58 | 0.64 | 0.48 | 0.50 | 0.08 | 0.49 | 0.46 |
| | trienoic | 0.99 | 0.78 | 0.64 | 1.02 | 0.86 | 1.12 | 1.34 | 1.53 | 1.44 | 1.11 | 1.33 | 1.31 |
| | tetraenoic | 2.19 | 2.83 | 2.80 | 2.23 | 2.51 | 0.28 | 0.35 | 0.43 | 0.45 | 0.39 | 0.40 | 0.38 |
| | pentaenoic | 1.08 | 1.64 | 1.53 | 1.05 | 1.33 | 0.05 | 0.09 | 0.10 | 0.09 | 0.05 | 0.11 | 0.08 |
| | hexaenoic | 1.33 | 1.84 | 1.91 | 1.40 | 1.62 | 0.17 | 0.27 | 0.44 | 0.17 | 0.20 | 0.20 | 0.24 |
| mg/g carcass | dienoic | 14.1 | 10.4 | 8.4 | 13.8 | 11.7 | 0.8 | 0.8 | 0.5 | 0.5 | 0.1 | 0.7 | 0.6 |
| | trienoic | 1.2 | 0.7 | 0.5 | 1.3 | 0.9 | 1.6 | 1.7 | 1.6 | 1.6 | 1.6 | 1.9 | 1.7 |
| | tetraenoic | 2.6 | 2.5 | 2.1 | 2.8 | 2.5 | 0.4 | 0.4 | 0.4 | 0.5 | 0.6 | 0.6 | 0.5 |
| | pentaenoic | 1.3 | 1.5 | 1.1 | 1.3 | 1.3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 |
| | hexaenoic | 1.6 | 1.6 | 1.4 | 1.8 | 1.6 | 0.2 | 0.3 | 0.4 | 0.2 | 0.3 | 0.3 | 0.3 |

[1] Including the non-saponifiable matter, the amount of which is considered negligible for the present purpose.

The absolute amounts of the individual polyenoic fatty acids exhibit a decrease in dienoic of 345 mg, *i. e.* to less than $^1/_4$ of the original value. Trienoic shows an increase of 259 mg, *i. e.* to more than 8 times the original value. Pentaenoic has decreased 31 mg, whereas tetra- and hexaenoic have only decreased slightly.

As in the chick experiment it is concluded that the increase in trienoic must have come from dienoic.

The percentage of the individual polyenoic fatty acids calculated on the basis of the total fatty acids shows a considerable decrease in dienoic, a moderate increase in trienoic and marked decrease in tetra-, penta- and hexaenoic. Calculated per g carcass weight trienoic has also increased and all the others decreased.

The quantitative differences found between the chick and the rat experiments are believed largely to be due to the differences in the length of the "fat-free" period. The shorter duration of the chick experiment was chosen because of the difficulties in analyzing the whole body of larger chicks. Had the chicken experiment run for a longer time it is likely that the quantitative results would have been closer to those found in the rat experiment.

However, in both experiments the absolute increase in trienoic acid must have the same cause, viz. conversion of dienoic to trienoic.

## Summary.

Chicks reared on a nearly fat-free diet for 4 weeks and rats reared on a fat-free diet for 18 weeks showed a considerable decrease of total dienoic fatty acid in the body. At the same time trienoic fatty acid increased markedly. The increase in trienoic fatty acid must have originated from dienoic since the changes of the other polyenoic fatty acids could not account for it.

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[1] Including the non-saponifiable matter, the amount of which is considered negligible for the present purpose.

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# Atrophy of Salivary Gland following Adrenalectomy or Hypophysectomy and the Effect of DOCA in Cats.

By

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In a study on cholinesterases recorded in the subsequent paper it was observed that in cats after hypophysectomy or removal of both adrenals the submaxillary gland looses in weight to an extent indicating atrophic changes. After the pioneer work by PH. E. SMITH in the rat (1930) it has been amply established in many species that various glandular structures manufacturing hormones for normal size and function depend on support from the pituitary gland. In this set of dependants the gastric and intestinal mucosa, potent producers of hormones, were included by the recent observation that in cats hypophysectomy or adrenalectomy is followed by an atrophy of the entire gastro-intestinal mucosa which in magnitude compares with the atrophy occurring in genuine endocrine organs (HAEGER, JACOBSOHN and KAHLSON 1952, 1953). The observations on the submaxillary gland were done in 1951—52 in the course of studies of the aforementioned atrophy and of the enzyme system histaminase.

## Material and Methods.

Adult cats of both sexes were used. They were killed by a blow on the head and exsanguinated. The submaxillary gland was removed and weighed to the next 5 mg. The right or left gland was chosen at random. The dry weight was determined by keeping the minced gland at 110° C until the weight was constant. The N-content of the dried

material was estimated by Kjeldahl's method. Observations were done on five groups of cats: 1) normal controls, 2) hypophysectomized, 3) adrenalectomized, 4) adrenalectomized maintained on DOCA, 5) cats on reduced food. The hypophysectomy was done by the parapharyngeal route and the adrenalectomy by the abdominal approach as described previously (HAEGER, JACOBSOHN and KAHLSON 1953). The adrenalectomized cats were kept at 25—28° C and given daily injections of saline under the skin. Group 4 received DOCA, 0.5—5.0 mg/kg, once daily, intramuscularly. In group 5 the quantity but not the composition of the daily food (milk, meat, fish, bread) was altered so as to achieve a loss in body weight of approximately 5 % per week, with a final reduction in weight of about 20 %.

## Results.

*Normal cats.* In 18 cats with a mean body weight of 2,600 $\pm$ 130 g the weight of one submaxillary gland was 1.17 $\pm$ 0.029 g and expressed in per cent of body weight 0.047 $\pm$ 0.003. In a different group of 19 cats referred to in a previous report from this institute the mean weights of the right and left submaxillary glands were 1.12 $\pm$ 0.054 and 1.14 $\pm$ 0.051 g, respectively (EMMELIN, JACOBSOHN and MUREN 1951).

*Hypophysectomized cats.* Individuals with inadequate appetite were stimulated to take additional meals from a dropfeeder. On this regime even at 33 and 56 days after the operation the cats, with three exceptions, did not loose in weight and were seemingly healthy.

The mean weight of the submaxillary gland in this group is 0.76 $\pm$ 0.077 g and expressed as per cent of body weight 0.027 $\pm$ 0.003 (Table 1). In this group with a mean body weight of 2,800 g against 2,600 g for the normal controls the weight of the whole gland is 35 % less than in the normal controls and 43 % less in per cent of body weight. The difference is statistically significant with $P < 0.001$ for both weight of the gland and per cent of body weight.

The bottom half of Table 1 comprises animals (cats 14—22) with minute remnants of hypophyseal tissue revealed on a few of the serial sections through the contents of the sella turcica and the infundibulum. With the exception of cat No. 22 the amount of specific tissue found in the remnants was certainly less than 5 % of the intact anterior lobe, and the cats of this group had more or less atrophic reproductive organs. The mean body weight in this group is 24 % greater than in the normal

**Table 1.**

*Cats No. 1—13 completely hypophysectomized, No. 14—22 have minute remnants.*

| No. and sex | Body weight, kg at op. | Body weight, kg at death | Days after hyp. ect. | Remnant of ant. lobe | Submax. gland weight in g | Submax. gland per cent of body wt. |
|---|---|---|---|---|---|---|
| 1 ♀ | 2.4 | 2.4 | 5 | 0 | 0.47 | 0.020 |
| 2 ♀ | 2.7 | 2.7 | 6 | 0 | 0.49 | 0.018 |
| 3 ♀ | 2.3 | 2.1 | 7 | 0 | 0.47 | 0.022 |
| 4 ♂ | 2.5 | 2.3 | 10 | 0 | 0.31 | 0.013 |
| 5 ♂ | 3.7 | 3.3 | 17 | 0 | 0.99 | 0.030 |
| 6 ♂ | 3.0 | 3.0 | 20 | 0 | 1.17 | 0.038 |
| 7 ♂ | 3.6 | 3.2 | 20 | 0 | 0.70 | 0.022 |
| 8 ♀ | 2.4 | 2.6 | 23 | 0 | 0.92 | 0.035 |
| 9 ♀ | 3.2 | 3.4 | 27 | 0 | 0.89 | 0.026 |
| 10 ♂ | 3.2 | 3.2 | 28 | 0 | 0.73 | 0.023 |
| 11 ♂ | 3.2 | 2.7 | 30 | 0 | 0.84 | 0.031 |
| 12 ♂ | 2.5 | 2.6 | 33 | 0 | 1.20 | 0.045 |
| 13 ♀ | 2.9 | 3.2 | 56 | 0 | 0.64 | 0.023 |
| | $2.9 \pm 0.13$ n = 13 | $2.8 \pm 0.12$ n = 13 | | | $0.76 \pm 0.077$ n = 13 | $0.027 \pm 0.003$ n = 13 |
| 14 ♀ | 3.2 | 3.0 | 21 | ((+)) | 1.30 | 0.043 |
| 15 ♂ | 3.5 | 3.3 | 27 | ((+)) | 1.71 | 0.052 |
| 16 ♂ | 3.1 | 3.3 | 15 | (+) | 1.30 | 0.039 |
| 17 ♀ | 2.5 | 2.5 | 21 | (+) | 0.90 | 0.038 |
| 18 ♂ | 3.6 | 3.5 | 22 | (+) | 1.22 | 0.035 |
| 19 ♂ | 3.8 | 3.7 | 30 | (+) | 1.40 | 0.038 |
| 20 ♀ | 3.3 | 3.1 | 20 | + | 0.87 | 0.028 |
| 21 ♂ | 3.1 | 3.1 | 24 | + | 0.87 | 0.028 |
| 22 ♀ | 2.9 | 3.2 | 17 | +++ | 1.21 | 0.038 |
| | $3.2 \pm 0.13$ n = 9 | $3.2 \pm 0.11$ n = 9 | | | $1.20 \pm 0.09$ n = 9 | $0.038 \pm 0.003$ n = 9 |

controls. The mean weight of the submaxillary gland is $1.20 \pm 0.09$ g and expressed as per cent of body weight $0.038 \pm 0.003$. This latter figure is significantly 19 % smaller than in normal controls ($P < 0.05$). In this group the reduction in submaxillary gland weight is smaller than in the cats without hypophyseal remnants.

As to the time course of the weight loss of the submaxillary gland after hypophysectomy the observations summarized in Table 1 give no definite information. The main loss seems to have occurred within 5 days, the shortest interval between operation and death in this series. In this connection it is noteworthy that the atrophy of the gastro-intestinal mucosa referred to above is fully established at five days after hypophysectomy and remained fairly constant during four weeks.

**Table 2.**

*Adrenalectomized cats.*

The bottom figures in brackets are means of cats No. 12—28 examined 4 days or later after operation.

| No. and sex | Body weight, kg | | Days after adr.ect. | Submax. gl. weight in g | Submax. gl. per cent of body wt. |
|---|---|---|---|---|---|
| | at op. | at death | | | |
| 1 ♂ | 2.4 | 2.4 | 1 | 0.79 | 0.033 |
| 2 ♂ | 3.6 | 3.4 | 2 | 1.02 | 0.030 |
| 3 ♀ | 3.0 | 2.9 | 2 | 0.81 | 0.028 |
| 4 ♀ | 3.4 | 3.3 | 2 | 1.32 | 0.040 |
| 5 ♀ | 2.0 | 2.0 | 2 | 0.84 | 0.043 |
| 6 ♀ | 2.3 | 2.3 | 2 | 0.72 | 0.031 |
| 7 ♂ | 2.3 | 2.3 | 2 | 0.58 | 0.025 |
| 8 ♀ | 2.1 | 2.1 | 3 | 0.89 | 0.043 |
| 9 ♂ | 2.3 | 2.0 | 3 | 0.71 | 0.035 |
| 10 ♀ | 2.2 | 2.2 | 3 | 1.37 | 0.063 |
| 11 ♀ | 2.9 | 2.7 | 3 | 0.80 | 0.030 |
| 12 ♀ | 3.5 | 3.5 | 4 | 1.09 | 0.031 |
| 13 ♂ | 3.4 | 3.2 | 4 | 1.02 | 0.032 |
| 14 ♀ | 2.7 | 2.7 | 4 | 1.15 | 0.043 |
| 15 ♀ | 3.4 | 3.2 | 4 | 1.06 | 0.033 |
| 16 ♀ | 2.5 | 2.5 | 5 | 0.69 | 0.028 |
| 17 ♀ | 1.8 | 1.7 | 5 | 0.68 | 0.041 |
| 18 ♂ | 2.3 | 2.1 | 5 | 1.09 | 0.052 |
| 19 ♀ | 2.2 | 2.1 | 5 | 1.00 | 0.048 |
| 20 ♂ | 3.2 | 3.1 | 5 | 0.83 | 0.027 |
| 21 ♂ | 3.7 | 3.4 | 5 | 0.85 | 0.025 |
| 22 ♀ | 3.3 | 3.1 | 6 | 0.99 | 0.032 |
| 23 ♀ | 3.0 | 2.9 | 6 | 1.10 | 0.038 |
| 24 ♀ | 2.4 | 2.1 | 7 | 0.61 | 0.029 |
| 25 ♂ | 3.4 | 3.2 | 8 | 0.91 | 0.029 |
| 26 ♀ | 2.7 | 2.3 | 11 | 0.51 | 0.022 |
| 27 ♂ | 3.1 | 2.6 | 12 | 0.59 | 0.023 |
| 28 ♂ | 3.1 | 2.6 | 12 | 0.50 | 0.019 |
| | $2.8 \pm 0.10$ n = 28 | $2.6 \pm 0.10$ n = 28 | | $0.88 \pm 0.043$ n = 28 | $0.034 \pm 0.002$ n = 28 |
| | $(2.9 \pm 0.13)$ n = 17 | $(2.7 \pm 0.13)$ n = 17 | | $(0.86 \pm 0.055)$ n = 17 | $(0.032 \pm 0.002)$ n = 17 |

Baker and Abrams (1955) observed in hypophysectomized rats that the weight of the parotid and submandibular glands is considerably reduced as compared with normal controls, the atrophy being more pronounced in the parotid gland. The absolute weight of the submandibular gland was significantly less in hypophysectomized rats but when considered in relation to body weight the difference was not significant. In these experiments on hypophysectomized rats treatment with growth hormone increased the weight of the gland in proportion to the gain in body weight, while cortisone acetate was effective in increasing the gland weight/body weight ratio.

**Table 8.**

*Adrenalectomized cats treated with DOCA.*

| No. and sex | Body weight, kg | | Days after adr.ect. | Submax. gl. weight in g | Submax. gl. per cent of body wt. | DOCA mg/per kg |
|---|---|---|---|---|---|---|
| | at op. | at death | | | | |
| 1 ♀ | 2.3 | 2.2 | 4 | 1.03 | 0.047 | 5.0 |
| 2 ♀ | 2.7 | 2.2 | 6 | 0.88 | 0.040 | 1.0 |
| 3 ♀ | 2.2 | 2.1 | 6 | 0.80 | 0.038 | 0.5 |
| 4 ♀ | 2.1 | 2.0 | 6 | 0.78 | 0.037 | 0.5 |
| 5 ♀ | 2.2 | 2.1 | 6 | 0.73 | 0.035 | 1.0 |
| 6 ♀ | 2.9 | 2.8 | 7 | 0.70 | 0.025 | 0.5 |
| 7 ♂ | 2.7 | 2.7 | 7 | 0.94 | 0.035 | 0.5 |
| 8 ♀ | 3.4 | 3.0 | 12 | 1.25 | 0.042 | 0.5 |
| 9 ♂ | 3.2 | 3.2 | 15 | 1.02 | 0.032 | 0.5 |
| 10 ♂ | 3.5 | 3.1 | 16 | 1.23 | 0.040 | 0.5 |
| | $2.7 \pm 0.16$ n = 10 | $2.5 \pm 0.15$ n = 10 | | $0.93 \pm 0.063$ n = 10 | $0.037 \pm 0.002$ n = 10 | |

It may be mentioned that the feline submaxillary gland after parasympathetic preganglionic denervation looses about 30 % in weight after two weeks (STRÖMBLAD 1955).

*Adrenalectomized cats.* Even with frequent additional meals from the dropfeeder the body weight at death in this group was slightly less than at the day of operation. The mean weight of the salivary glands is $0.88 \pm 0.043$ g and $0.034 \pm 0.002$ in per cent of body weight. In the whole group the mean weight of the gland in per cent of body weight is 29 % less than in the normal controls. In the group examined 4 days after the operation or later (Nos. 12—28) the weight of the gland is 32 % less than in the normal controls. In three cats examined at 11 and 12 days after the operation the loss in weight of the salivary gland is about 50 % (Table 2).

Although the cats were given daily injections of saline the possibility of dehydration as contributing to the loss in salivary gland weight in this group must be considered. To assess this possibility dry weight and total N was determined in submaxillary glands from 5 adrenalectomized and 10 normal cats. In the adrenalectomized group the dry weight was 25 % within a very narrow range and 11.2 % for total N in the dried residue. In the control group the corresponding values are 22 % and 11.3 %. The slight dehydration of the gland after adrenalectomy obviously does not contribute substantially to the loss in submaxillary gland weight.

*Adrenalectomized cats maintained on DOCA.* A group of ten adrenalectomized cats were given daily doses of DOCA, 0.5

**Table 4.**
*Cats on reduced food.*

| No. and sex | Body weight, kg at the beginning | Body weight, kg after 4 weeks on reduced food | Loss in weight per cent | Submax. gland weight in g | Submax. gland per cent of body weight |
|---|---|---|---|---|---|
| 1 ♂ | 3.3 | 2.6 | 20 | 1.03 | 0.040 |
| 2 ♂ | 1.9 | 1.4 | 24 | 0.72 | 0.051 |
| 3 ♀ | 2.1 | 1.7 | 20 | 0.93 | 0.054 |
| 4 ♀ | 2.8 | 2.3 | 17 | 0.91 | 0.039 |
| 5 ♂ | 3.2 | 2.6 | 18 | 1.09 | 0.042 |
| 6 ♀ | 2.7 | 2.1 | 21 | 0.97 | 0.046 |
| | $2.7 \pm 0.23$ n = 6 | $2.1 \pm 0.20$ n = 6 | 20 | $0.94 \pm 0.52$ n = 6 | $0.045 \pm 0.003$ n = 6 |

—5 mg/kg per body weight (Table 3). In this group the mean weight of the submaxillary gland expressed as per cent of body weight is 16 % greater than in the corresponding non treated adrenalectomized group of Table 2 examined 4 days or later after operation ($0.05 < P < 0.1$). — Further experiments with large doses of DOCA seem justified.

*Cats on reduced food.* Since the adrenalectomized cats as a group lost about 7 % in body weight observations were made on cats with restricted food intake. After four weeks this group had lost about 20 % in body weight. The values for the salivary glands are $0.94 \pm 0.052$ g and $0.045 \pm 0.03$ per cent, respectively (Table 4) as compared with $0.047 \pm 0.003$ for normal cats. Expressed in per cent of body weight the salivary gland in this group has the same weight as in normal controls, whereas the weight of the total gland is reduced to about the same extent as the total body weight. It should be noted that in this group the loss in body weight is considerably greater than in the adrenalectomized cats.

## Comments.

Ever since the discovery of Ph. E. SMITH, who spoke of a "splanchnomicria", it is well established that after hypophysectomy various glands and viscera undergo atrophy. The loss in weight is most pronounced in structures subjected to what is referred to as the trophic influence of the pituitary gland, while in "non-target" organs, such as the liver and the heart, the loss in weight is associated with the reduction in general metabolism and body weight following hypophysectomy.

The weight of the heart in hypophysectomized rats is 12 % below normal (BEZNAK 1954 a). Treatment of hypophysectomized rats with ACTH or cortisone has no influence on the loss of cardiac weight after hypophysectomy, whereas growth hormone will abolish cardiac atrophy (BEZNAK 1954 a). The cardiac atrophy after hypophysectomy is relatively smaller than the reduction in general metabolism observed by various authors after hypophysectomy.

The liver does not loose weight in rats after hypophysectomy if feeding is such as to prevent loss in total body weight (RINALDINI 1950, CHAN 1950).

The pancreas of rats looses considerably in weight in undernutrition, and is reduced to about half the normal size after hypophysectomy. The atrophy affects the exocrine components as judged by the decrease in amylase activity (BARRETT, NISHIKAWARA and HAIST 1955). These authors discuss the possibility that the changes in the pancreas may be due to changes in some pancreas stimulating hormone(s) under pituitary control.

The supporting influence of the pituitary on the salivary gland seems to be mediated by the adrenals, the removal of which consistently causes a reduction of the submaxillary gland, in some instances to less than half the initial weight.

It is noteworthy that treatment with DOCA possibly has a slight effect in preventing the atrophy of the salivary gland. Larger doses than those so far tried in the present experiments may be more effective. This brings to mind the observation that in the hypophysectomized rat large doses of DOCA, 5—10 mg per rat, diminish cardiac atrophy (BEZNAK 1954 b).

The salivary gland, thus, in conjunction with the gastrointestinal mucosa, ranks, beside the well established target organs, among the structures which for normal size depend on support from the pituitary gland. This similarity raises the question whether the salivary gland, like the fellow-dependants, is endowed with endocrine function. Such status has, in fact, been claimed by various authors. The evidence, however, is confusing and has been reviewed by BABKIN (1950). A report by HÄMMERLI (1920), not quoted by BABKIN, seems pertinent: HÄMMERLI observed in a man at autopsy a striking hypertrophy of the parotid and submaxillary glands associated with hypertrophy of the adrenal glands. More recently it has been claimed that the salivary glands play a rôle in the extrathyroidal metabolism of organic iodine

and that these function as "reverse thyroids" (FAWCETT and KIRKWOOD 1954, RYAN and KIRKWOOD 1955). As to the atrophic salivary gland in the first place studies of its microstructure and exocrine function seem desirable.

## Summary.

1. The weight of the submaxillary gland was determined in five groups of cats: 1) normal, 2) hypophysectomized, 3) adrenalectomized, 4) adrenalectomized maintained on DOCA, 5) undernourished.

2. Hypophysectomized adult cats, properly fed, can be kept for several weeks without loss in body weight. Adrenalectomized adult cats, with proper feeding and saline injections, can be kept up to 16 days with a loss in body weight of only 4—10 %.

3. After hypophysectomy the submaxillary gland looses about 40 % in weight, after adrenalectomy the gland may be reduced to less than half the original size. Dehydration does not essentially contribute to this change.

4. DOCA in daily doses of 0.5—5.0 mg/kg possibly has a slight effect in preventing the atrophy of the salivary gland in adrenalectomized cats.

5. In undernutrition, resulting in 20 % loss in body weight in four weeks, the weight of the salivary gland in per cent of body weight is the same as in normal controls.

Hypophysectomy and control of completeness of the operation were done by Dr. DORA JACOBSOHN, whose help we gratefully acknowledge.

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# The Distribution of Cholinesterases in Cats and Changes Caused by Hypophysectomy, Adrenalectomy, Undernutrition and DOCA.

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The experiments to be reported were done as a corollary to studies in this institute on the enzyme system histaminase. It was noted that in cats hypophysectomy as well as adrenalectomy is followed by a profound depletion of the histaminase depots. After either of these operations 70—80 % of the extractable histaminase activity disappears from the kidneys and intestinal mucosa. This change takes place rapidly after adrenalectomy. Hypophysectomy is followed by similar changes in histaminase, although at a slower time course, the final depletion of the stores being of the same magnitude as after adrenalectomy. From this it was inferred that the specific adrenocortical secretion essential to normal histaminase metabolism is entirely dependent on the pituitary gland. These changes in histaminase can be prevented or restored by administration of adrenal cortical extract or cortisone, but not by DOCA (CARLSTEN and WOOD 1951, HAEGER, JACOBSOHN and KAHLSON 1952, 1953, KAHLSON, LINDELL and WESTLING 1953, HAEGER 1953, for review see KAHLSON 1956).

It seemed of interest to establish, in the series of cats studied for histaminase, whether other enzymes functionally related to "local hormones" are, like histaminase, subjected to pituitary-adrenal control. In the experiments on cholinesterases we have, so far, only examined the gastro-intestinal mucosa, the liver and the submaxillary gland.

For the sake of simplicity and practical usefulness we adhere in terminology to Mendel's distinction of a "true" and a "pseudo"

cholinesterase, specifically hydrolyzing respectively acetyl-β-methylcholine and benzoylcholine (or butyrylcholine) (for review see WHITTAKER 1951).

KOELLE and his colleagues noted in the cat by a histochemical technique the presence of true cholinesterase throughout the parenchyma of the parotid gland, in the liver they found the nuclei of the hepatic cells stained for non-specific cholinesterase activity, while in the liver no significant concentration of specific cholinesterase activity could be detected. In the cat's ileum these workers found both types of enzyme in the nervous structures of the myenteric and submucous plexuses, specific cholinesterase in the circular muscle and in the muscle fibers and epithelial cells of the villi, non-specific cholinesterase in the longitudinal muscle and the muscularis mucosae (KOELLE 1950, KOELLE, KOELLE and FRIEDENWALD 1950). ORD and THOMPSON (1950) investigated the distribution of cholinesterases in the rat and found that benzoylcholine and acetyl-β-methylcholine are hydrolyzed at approximately equal rates by stomach, liver, lung and submaxillary gland, while in the case of heart auricle and ventricle, intestinal muscle and mucosa, Harderian gland, and skin, benzoylcholine is hydrolyzed more rapidly than acetyl-β-methylcholine. In experiments on enzyme summation and inhibition they established that in the rat these tissues contain two cholinesterases, the "true" and "pseudo" esterase in Mendel's sense. — In the cat HELLMANN using histochemical methods noted cholinesterase activity at the motor endplates of the diaphragm (1952) and at the nerve endings surrounding the sweat glands of the hairless pads (1955).

## Experimental.

Most of the experiments were done in all seasons of the years 1951—1952. The cats are the same as those used in the preceding report on the atrophy of the salivary gland. In the course of the experiments with cholinesterases it was found that with gastro-intestinal mucosa and liver the esterases could be extracted with a yield of about 100 %, the "true" type in the intestine being somewhat less extractable than the other, whereas with the submaxillary gland the extract contained only 25—40 % of the activity as compared with the homogenate. Both extracts and homogenates were assayed.

*Preparation of the homogenate.* Immediately after death the tissues under investigation were removed, weighed and then kept frozen at — 20° C until the next day. The tissue specimens were then minced with scissors and homogenized with a Potter-Elvehjem homogenizer in a buffer solution according to AUGUSTINSSON (1949): 0.15 M NaCl, 0.025

M $NaHCO_3$ and 0.04 M $MgCl_2$—$6H_2O$. To one part of the salivary gland were added 4 parts of the buffer solution. For the liver and gastric mucosa the proportion was 1.0 g and 6.0 ml and for intestinal mucosa 1.0 g and 2.5 ml. The mixture was kept cold during homogenization. For each estimation 0.1 ml was brought into the Warburg vessel.

*Preparation of the extract.* The homogenate was centrifuged for 5 min. at 2,000 × gravity, the supernatant fluid decanted and assayed for cholinesterase activity. This supernatant is referred to as the extract, 0.3 ml of which was used for each estimation.

*Estimation of esterase activity.* The Warburg manometric technique in AMMON's (1933) modification was used. The measurements were made at 37° C. Gas phase: 95 % $N_2$ and 5 % $CO_2$ by volume. Readings were made every 6 minutes. Esterase activity is expressed in $b_{30}$ values, *i. e.* µl $CO_2$/g of tissue evolved during 30 min. minus the corresponding value for non-enzymic hydrolysis (AUGUSTINSSON 1948).

*Substrates.* The chlorides of the following esters were used: 1) acetylcholine (ACh); 2) dl-acetyl-β-methylcholine (MCh); 3) butyrylcholine (BuCh), which is a more specific substrate for "pseudo" esterase than benzoylcholine (for discussion see AUGUSTINSSON 1949). The concentrations of the substrates are given in the tables. Each substrate was dissolved immediately before use in a buffer solution of the same composition as was used in the preparation of the enzymes.

*Statistics.* Figures summarizing a series of observations are given as mean ± standard error of mean; n = number of observations.

## Results.

### Normal Cats.

*Submaxillary gland.* The relative esterase activity, $b_{30}$, of extract and homogenate was estimated and expressed as activity per gram tissue as well as for the whole gland. Table 1 includes six cats where in one and the same gland the homogenate as well as the extract was assayed. It will be noted that in the submaxillary gland only approximately 30 % of the total enzyme activity is extractable. The "true" form is less extractable than the "pseudo" form which is apparent from the MCh/BuCh ratio.

These figures are in accord with STRÖMBLAD (1955) who with the esterases in the cat's submaxillary gland found a high activity against ACh and a low towards BuCh. In a series of 10 cats he found in the homogenate a mean activity towards ACh of 3,291 ± 470 per g tissue (unpublished observations in this institute). DIRNHUBER and EVANS (1954) determined the cholinesterase content in homogenates of the submaxillary gland in 5 cats.

*Liver.* Determinations in 14 normal cats are included in Table 2. The esterase activity is easily extractable. The activity towards

**Table 1.**

*Normal cats. Cholinesterase activities in homogenates and extracts of salivary gland as $b_{30}$ per g tissue and (in brackets) in the whole gland. The per cent figures denote activity in extract as compared with homogenate.*

| No. and sex | Activity | | | | | | MCh/BuCh |
|---|---|---|---|---|---|---|---|
| | ACh $3.3 \cdot 10^{-3}$ M | | MCh $3.1 \cdot 10^{-2}$ M | | BuCh $2.9 \cdot 10^{-3}$ M | | |
| 1. Homog. | 2,870 (2.181) | | 1,540 (1,170) | | 630 (479) | | 2.44 |
| ♀Extract | 817 (621) | 29% | 397 (671) | 26% | 187 (142) | 30% | 2.12 |
| 2. Homog. | 4,060 (3.573) | | 2,310 (2,033) | | 910 (801) | | 2.54 |
| ♀Extract | 933 (821) | 23% | 467 (411) | 20% | 233 (205) | 26% | 2.00 |
| 3. Homog. | 3,010 (3.823) | | 1,120 (1.422) | | 490 (622) | | 2.30 |
| ♂Extract | 840 (1,067) | 28% | 373 (474) | 33% | 232 (296) | 48% | 1.60 |
| 4. Homog. | 3,010 (2.829) | | 1,750 (1.645) | | 700 (658) | | 2.50 |
| ♂Extract | 793 (745) | 26% | 327 (307) | 19% | 187 (176) | 27% | 1.75 |
| 5. Homog. | 3,450 (5.831) | | 1.400 (2.366) | | 1,100(1.859) | | 1.27 |
| ♂Extract | 1,167 (1,972) | 34% | 533 (901) | 38% | 400 (676) | 36% | 1.33 |
| 6. Homog. | 3.833 (5.941) | | 1,550 (2.403) | | 800(1.240) | | 1.94 |
| ♀Extract | 1,233 (1,911) | 32% | 483 (749) | 31% | 317 (491) | 40% | 1.52 |
| Homog. | $3,372 \pm 201$ ($4,030 \pm 633$) | | $1,612 \pm 164$ ($1,840 \pm 208$) | | $772 \pm 88$ ($943 \pm 212$) | | $2.17 \pm 0.20$ |
| Extract | $964 \pm 78$ ($1,190 \pm 245$) n = 6 | 29% | $430 \pm 31$ ($586 \pm 92$) n = 6 | 28% | $260 \pm 34$ ($331 \pm 86$) n = 6 | 35% | $1.72 \pm 0.12$ n = 6 |

MCh was too low to be properly estimated. With the methods used there is no evidence of the presence of "true" cholinesterase in the cat's liver.

*Gastric mucosa.* The mucosa from all regions of the stomach was pooled and examined in 10 normal cats. The figures are given in Table 3. In homogenized gastric mucosa the concentration of "true" cholinesterase is at about the threshold level which can be assessed by the methods used. It will be seen from Table 3 that the "pseudo" esterase activity can be easily extracted from the gastric mucosa. The relative activities against the various substrates established in cats are about the same as recorded by ORD and THOMPSON for the stomach in 2 rats.

*Small intestine.* The mucosa from all regions of the small intestine was pooled and examined in 12 normal cats. The values are given in Table 4. As judged by the ratio MCh/BuCh "true" cholinesterase is present in considerable amounts.

**Table 2.**

*Cholinesterase activities in homogenates and extracts of liver of normal, hypophysectomized and adrenalectomized cats. The per cent figures denote activity in extract as compared with homogenate.*

| No. and sex | Days after oper. | Activity | | | | | |
|---|---|---|---|---|---|---|---|
| | | ACh $3.3 \cdot 10^{-3}$ M | | | BuCh $2.9 \cdot 10^{-3}$ M | | |
| | | Homog. | Extract | | Homog. | Extract | |
| Normal | | | | | | | |
| 1—11 ..... | — | | 1,616 ± 127 | | | 4,960 ± 379 | |
| 12 ♂ ..... | — | 2,240 | 2,065 | 92 % | | | |
| 13 ♀ ..... | — | 4,445 | 3,640 | 82 % | 6,930 | 7,000 | 101 % |
| 14 ♂ ..... | — | 1,715 | 1,715 | 100 % | | | |
| | | | 1,800 ± 176<br>n = 14 | | | 5,130 ± 386<br>n = 12 | |
| Hyp.ect. | | | | | | | |
| 3 ♂ ..... | 20 | | 1,925 | | | 5,845 | |
| 5 ♀ ..... | 27 | | 2,205 | | | 6,790 | |
| 6 ♂ ..... | 28 | | 980 | | | 2,240 | |
| 9 ♀ ..... | 56 | | 1,190 | | | 3,080 | |
| | | | 1,575 ± 292<br>n = 4 | | | 4,489 ± 344<br>n = 4 | |
| Adren.ect. | | | | | | | |
| 5 ♀ ..... | 2 | | 875 | | | 2,275 | |
| 6 ♀ ..... | 2 | | 1,715 | | | 6,405 | |
| 7 ♂ ..... | 2 | | 1,540 | | | 5,250 | |
| 8 ♀ ..... | 3 | | 2,485 | | | 7,000 | |
| 9 ♂ ..... | 3 | | 2,200 | | | 6,925 | |
| 11 ♀ ..... | 3 | 2,555 | 2,450 | 96 % | 5,320 | 4,690 | 88 % |
| 12 ♀ ..... | 4 | 2,625 | 2,450 | 93 % | 7,140 | 7,140 | 100 % |
| 13 ♂ ..... | 4 | 2,380 | 2,380 | 100 % | | | |
| 30 ♀ ..... | 4 | | 1,295 | | | 3,570 | |
| 31 ♀ ..... | 4 | | 1,260 | | | 4,060 | |
| 16 ♂ ..... | 5 | 1,645 | 1,453 | 87 % | 4,655 | 4,305 | 92 % |
| 18 ♂ ..... | 5 | | 2,275 | | | 8,750 | |
| 33 ♀ ..... | 6 | 3,745 | 3,745 | 100 % | | | |
| 24 ♀ ..... | 7 | | 1,575 | | | 4,340 | |
| 27 ♂ ..... | 12 | | 1,750 | | | 4,515 | |
| | | | 1,962 ± 58<br>n = 15 | | | 5,325 ± 497<br>n = 13 | |

*Characteristics of various types of cholinesterases.* Characteristic of "true" cholinesterase, referred to by AUGUSTINSSON and NACHMANSOHN as acetylcholinesterase, is the depression of its activity by high concentrations of ACh and its specificity against MCh, whereas this type of esterase hydrolyzes BuCh at a low rate only.

**Table 3.**

*Cholinesterase activities in homogenates and extracts of gastric mucosa of normal, hypophysectomized and adrenalectomized cats. The per cent figures denote activity in extract as compared with homogenate.*

| No. and sex | Days after oper. | Activity | | | | | |
|---|---|---|---|---|---|---|---|
| | | ACh $3.3 \cdot 10^{-3}$ M | | | BuCh $2.9 \cdot 10^{-3}$ M | | |
| | | Homog. | Extract | | Homog. | Extract | |
| Normal | | | | | | | |
| 1—7 ..... | | | $2{,}606 \pm 169$ n = 7 | | | $5{,}443 \pm 433$ n = 6 | |
| 8 ♂ ..... | | 2,765 | 2,695 | 97 % | | | |
| 9 ♀ ..... | | 1,680 | 1,365 | 81 % | 4,830 | 4,130 | 86 % |
| 10 ♂ ..... | | 3,570 | 2,940 | 82 % | | | |
| | | | $2{,}524 \pm 176$ n = 10 | | | $5{,}255 \pm 411$ n = 7 | |
| Hyp.ect. | | | | | | | |
| 3 ♂ ..... | 20 | | 1,295 | | | 3,680 | |
| 21 ♂ ..... | 23 | | 3,150 | | | 7,630 | |
| 5 ♀ ..... | 27 | | 2,202 | | | 4,585 | |
| 6 ♂ ..... | 28 | | 2,870 | | | 5,285 | |
| 9 ♀ ..... | 56 | | 2,485 | | | 6,125 | |
| | | | $2{,}400 \pm 320$ n = 5 | | | $5{,}461 \pm 675$ n = 5 | |
| Adr.ect. | | | | | | | |
| 6 ♀ ..... | 2 | | 3,290 | | | | |
| 7 ♂ ..... | 2 | | 1,995 | | | 3,150 | |
| 9 ♂ ..... | 3 | | 3,080 | | | 8,575 | |
| 10 ♀ ..... | 3 | | 1,470 | | | 2,030 | |
| 11 ♀ ..... | 3 | 3,045 | 2,695 | 89 % | 4,060 | 3,920 | 97 % |
| 12 ♀ ..... | 4 | 4,515 | 3,920 | 87 % | 7,630 | 6,930 | 91 % |
| 16 ♂ ..... | 5 | 3,780 | 3,745 | 99 % | 6,510 | 6,650 | 102 % |
| 18 ♂ ..... | 5 | | 2,590 | | | 5,320 | |
| 33 ♀ ..... | 6 | 3,045 | 2,870 | 94 % | | | |
| 29 ♂ ..... | 6 | | 2,975 | | | 7,385 | |
| 26 ♀ ..... | 11 | | 2,420 | | | | |
| | | | $2{,}823 \pm 216$ n = 11 | | | $5{,}495 \pm 806$ n = 8 | |

By contrast, the "pseudo" type readily hydrolyzes BuCh and its activity against ACh is not inhibited by high concentrations of this substrate. On this view the types of esterase activity in the submaxillary gland and intestinal mucosa are represented in Fig. 1 and from gastric mucosa and liver in Fig. 2. The enzyme activity in the extracts from these tissues was determined using various

*Cholinesterase activities expressed as $dS^{\circ}_{30}$ per g tissue in homogenates and extracts of intestinal mucosa of normal, hypophysectomized and adrenalectomized cats. The per cent figures denote activity in extract as compared with homogenate.*

Activity

Days

*Cholinesterase activities expressed as $b_{30}$ per g tissue in homogenates and extracts of intestinal mucosa of normal, hypophysectomized and adrenalectomized cats. The per cent figures denote activity in extract as compared with homogenate.*

| No. and sex | Days after oper. | Activity | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ACh $3.3 \cdot 10^{-3}$ M | | | MCh $3.1 \cdot 10^{-3}$ M | | | BuCh $2.9 \cdot 10^{-3}$ M | | | MCh/BuCh | |
| | | Homog. | Extract | | Homog. | Extract | | Homog. | Extract | | Homog. | Extract |
| Normal | | | | | | | | | | | | |
| 1—9 .... | | | 623 ± 79<br>n = 9 | | | 245 ± 40<br>n = 9 | | | 340 ± 59<br>n = 8 | | | 0.88<br>n = 8 |
| 10 ♂ ..... | | 742 | 617 | 83 % | | | | | | | | |
| 11 ♀ ..... | | 760 | 573 | 75 % | 293 | 213 | 73 % | 413 | 373 | 90 % | 0.71 | 0.57 |
| 12 ♂ ..... | | 493 | 387 | 78 % | 253 | 187 | 74 % | 267 | 253 | 95 % | 0.95 | 0.74 |
| | | | 599 ± 62<br>n = 12 | | | 237 ± 33<br>n = 11 | | | 335 ± 47<br>n = 10 | | | 0.83±0.1<br>n = 10 |
| Hyp.ect. | | | | | | | | | | | | |
| 3 ♂ ..... | 20 | | 453 | | | 226 | | | 213 | | | 1.06 |
| 21 ♂ ..... | 23 | | 700 | | | 175 | | | 245 | | | 0.71 |
| 5 ♀ ..... | 27 | | 560 | | | 240 | | | 350 | | | 0.69 |
| 6 ♂ ..... | 28 | | 570 | | | | | | 220 | | | |
| 9 ♀ ..... | 56 | | 450 | | | 200 | | | 210 | | | 0.95 |
| | | | 547 ± 46<br>n = 5 | | | 210 ± 14<br>n = 4 | | | 248 ± 26<br>n = 5 | | | 0.85<br>n = 4 |
| Adr.ect. | | | | | | | | | | | | |
| 6 ♀ ..... | 2 | | 680 | | | 293 | | | 453 | | | 0.65 |
| 7 ♂ ..... | 2 | | 707 | | | 387 | | | 253 | | | 1.53 |
| 9 ♂ ..... | 3 | | 513 | | | | | | 238 | | | |
| 11 ♀ ..... | 3 | 1,040 | 880 | 85 % | 380 | 320 | 84 % | 380 | 420 | 110 % | 1.0 | 0.76 |
| 12 ♀ ..... | 4 | 1,747 | 1,747 | 100 % | | | | | | | | |
| 13 ♂ ..... | 4 | 1,100 | 1,060 | 96 % | | | | | | | | |
| 14 ♀ ..... | 4 | | 570 | | | | | | | | | |
| 16 ♂ ..... | 5 | 940 | 880 | 94 % | 360 | 340 | 94 % | 680 | 700 | 103 % | 0.53 | 0.49 |
| 18 ♂ ..... | 5 | | 466 | | | | | | 217 | | | |
| 33 ♀ ..... | 6 | 1,187 | 1,160 | 98 % | 360 | 333 | 93 % | | | | | |
| 27 ♂ ..... | 12 | | 948 | | | 340 | | | 481 | | | 0.71 |
| | | | 874 ± 111<br>n = 11 | | | 336 ± 13<br>n = 6 | | | 395 ± 66<br>n = 7 | | | 0.83 ± 0.18<br>n = 5 |

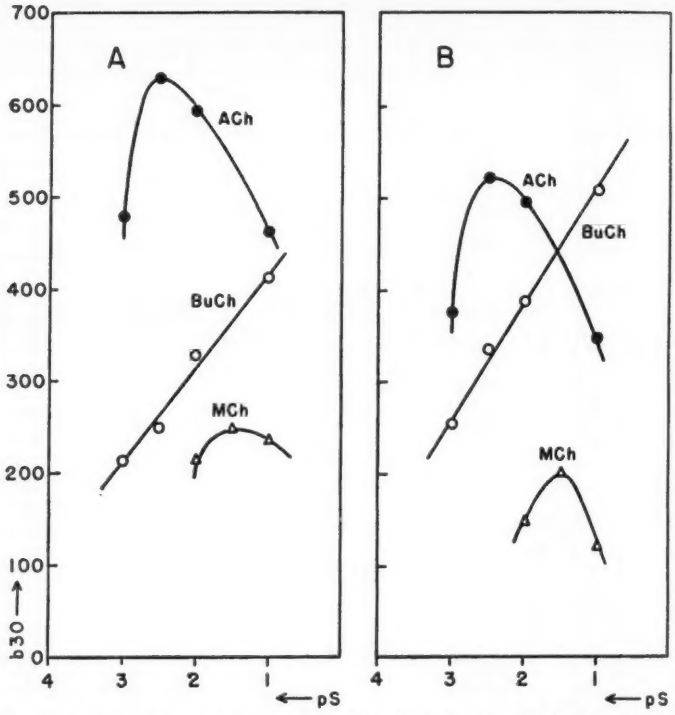


Fig. 1. Activity-pS curves for the enzymic hydrolysis of acetylcholine chloride (ACh); dl-acetyl-β-methylcholinechloride (MCh) and butyrylcholinechloride (BuCh) by extracts from glandula submaxillaris (A) and mucosa from the small intestine (B).

concentrations of the substrates. In the figures the enzyme activity is plotted against the negative logarithm of molar substrate concentration ($b_{30}$/pS). The activity against various concentrations of ACh in extracts from the submaxillary gland and intestinal mucosa shows a bellshaped curve with an optimal activity at about $3 \times 10^{-3}$ M ACh which is the same as noted by various authors with acetylcholinesterase in nervous tissue and erythrocytes. The hydrolysis of BuCh by the enzymes from the submaxillary gland is at a low rate compared with that of ACh. These facts indicate that the cholinesterase of the salivary gland is predominantly of the "true" type. The distinction between acetylcholinesterase and other cholinester splitting enzymes based on the ac-

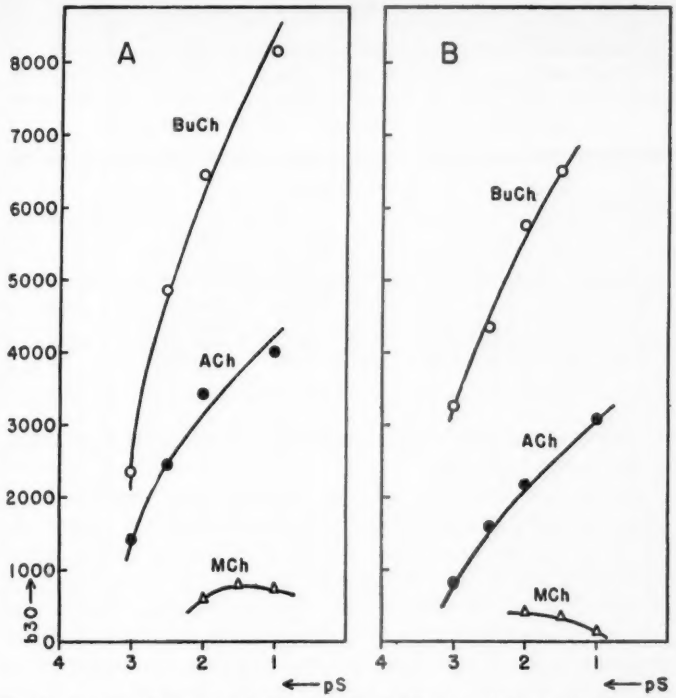


Fig. 2. Activity-pS curves as in Fig. 1 obtained with extracts from gastric mucosa (A) and liver (B).

tivity as function of substrate concentration was emphasized by AUGUSTINSSON (1948) and AUGUSTINSSON and NACHMANSOHN (1949).

### Hypophysectomized Cats.

*Submaxillary gland.* In 2 cats the gland of one side was removed at the operation of hypophysectomy, the gland of the other side 22 and 23 days later. After hypophysectomy the weight of the glands was reduced by 25 % and 38 %, respectively, and the activity of the extracts towards ACh increased by 74 % and 142 %, respectively. When at a later stage of the present experiments it was noted that the extract of the submaxillary gland contains considerably less esterase activity than the homogenate, the latter was examined in 4 hypophysectomized cats (Table 5). In 2 cats

**Table 5.**

*Hypophysectomized cats. Cholinesterase activities in homogenates of salivary gland expressed as $b_{30}$ per g tissue and (in brackets) in the whole gland. This group of cats, with their individual numbers, is identical with the corresponding group referred to in the preceding paper.*

| No. and sex | Days after hyp. ect. | Activity | | | MCh/BuCh |
|---|---|---|---|---|---|
| | | ACh $3.3 \cdot 10^{-3}$ M | MCh $3.1 \cdot 10^{-2}$ M | BuCh $2.9 \cdot 10^{-3}$ M | |
| 1 ♀ | 5 | 5,350 (2,515) | 2,700 (1,269) | 1,400 (658) | 1.93 |
| 2 ♀ | 6 | 5,175 (2,536) | 2,400 (1,176) | 1,575 (772) | 1.52 |
| 3 ♀ | 7 | 3,300 (1,551) | 1,850 (870) | 650 (306) | 2.85 |
| 4 ♂ | 10 | 3,400 (1,054) | 1,600 (496) | 900 (279) | 1.78 |

the activity towards all three substrates is significantly higher than in the normal controls.

*Gastro-intestinal mucosa and liver.* As seen from Tables 2, 3 and 4 the $b_{30}$ values per gram wet weight in the liver, the gastric and intestinal mucosa are the same as in normal cats. In this hypophysectomized group the gastric and intestinal mucosa was atrophic, in accord with earlier reports (HAEGER, JACOBSOHN and KAHLSON 1953). It is noteworthy that this atrophy which strikingly reduces the villus length, is without consequence as regards the relative esterase activity in the extracts, suggesting a rather uniform distribution of the esterases along the various layers of the mucosa.

### Adrenalectomized Cats.

In rats the cholinesterase activity after adrenalectomy has been investigated by various authors. STOERK and MORPETH (1944) reported a slight lowering of the "total choline esterase" of the whole gastrocnemic muscle after adrenalectomy, while OVERBEEK (1949) in the same muscle recorded an increase in activity. GREIG and CARTER (1954) determined the cholinesterase activity of brain, liver, plasma, erythrocytes and skeletal muscle of normal and adrenalectomized rats and, after adrenalectomy, found a consistent decrease in esterase activity in the kidney only. In vitro the decreased activity of the "true" cholinesterase could be restored by adrenal cortical extract and compound F but not by DOCA which, however, in vitro reactivated the "pseudo"

**Table 6.**

*Adrenalectomized cats. Cholinesterase activities in homogenates and extracts of salivary gland as $b_{30}$ per g tissue and (in brackets)*

**Table 6.**

*Adrenalectomized cats. Cholinesterase activities in homogenates and extracts of salivary gland as $b_{30}$ per g tissue and (in brackets) in the whole gland. The per cent figures denote activity in extract as compared with homogenate. This group of cats, with their individual numbers, is identical with the adrenalectomized group in the preceding paper.*

| No. and sex | Days after adr.ect. | Activity | | | | | | MCh/BuCh |
|---|---|---|---|---|---|---|---|---|
| | | ACh $3.3 \cdot 10^{-3}$ M | | MCh $3.1 \cdot 10^{-2}$ M | | BuCh $2.9 \cdot 10^{-3}$ M | | |
| 1. Homog. ..... | 1 | 4,690 (3,705) | | 2,520 (1,991) | | 770 (608) | | 3.27 |
| ♂ Extract ..... | | 1,400 (1,106) | 30 % | 560 (442) | 22 % | 280 (221) | 36 % | 2.00 |
| 2. Homog. ..... | 2 | 4,690 (4,784) | | 2,310 (2,356) | | 910 (928) | | 2.54 |
| ♂ Extract ..... | | 1,540 (1,571) | 33 % | 793 (809) | 34 % | 490 (500) | 54 % | 1.62 |
| 3. Homog. ..... | 2 | 4,900 (3,969) | | 2,590 (2,098) | | 1,960 (1,588) | | 1.32 |
| ♀ Extract ..... | | 2,030 (1,644) | 41 % | 863 (699) | 33 % | 700 (567) | 36 % | 1.23 |
| 4. Homog. ..... | 2 | 3,990 (5,267) | | 1,890 (2,495) | | 980 (1,294) | | 1.93 |
| ♀ Extract ..... | | 1,330 (1,756) | 33 % | 583 (770) | 31 % | | | |
| 11. Homog. ..... | 3 | 4,690 (3,752) | | 2,100 (1,680) | | 1,120 (896) | | 1.88 |
| ♀ Extract ..... | | 1,190 (952) | 25 % | 560 (448) | 27 % | 397 (318) | 35 % | 1.41 |
| 12. Homog. ..... | 4 | 6,020 (6,562) | | 2,660 (2,899) | | 1,120 (1,221) | | 2.38 |
| ♀ Extract ..... | | 1,727 (1,882) | 29 % | 770 (839) | 29 % | 537 (585) | 48 % | 1.43 |
| 13. Homog. ..... | 4 | 3,920 (3,998) | | 1,890 (1,928) | | 700 (714) | | 2.70 |
| ♂ Extract ..... | | 980 (1,000) | 25 % | 420 (428) | 22 % | 443 (452) | 63 % | 0.95 |
| 16. Homog. ..... | 5 | 5,520 (3,809) | | 2,829 (1,952) | | 1,725 (1,190) | | 1.64 |
| ♂ Extract ..... | | 2,208 (1,524) | 40 % | 966 (667) | 34 % | 874 (603) | 51 % | 1.11 |
| Homog. ..... | | 4,803 ± 249 (4,481 ± 357) | | 2,349 ± 127 (2,175 ± 137) | | 1,161 ± 160 (1,055 ± 115) n = 8 | | 2.21 ± 0.22 n = 8 |
| Extract ..... | | 1,551 ± 148 (1,429 ± 127) n = 8 | 32 % ± 2.2 n = 8 | 689 ± 66 (638 ± 61) n = 8 | 29 % ± 1.8 n = 8 | 532 ± 75 (464 ± 55) n = 7 | 46 % ± 4.1 n = 7 | 1.39 ± 0.13 n = 7 |

**Table 7**

*Cats on reduced food. Cholinesterase activity in extracts expressed as $b_{30}$ per g tissue for liver, the gastric and intestinal mucosa.*

| No. and sex | Body weight, kg | | Activity | | | MCh/BuCh |
|---|---|---|---|---|---|---|
| | at the beginning | after 4 weeks on reduced food | ACh $3.3 \cdot 10^{-3}$ M | MCh $3.1 \cdot 10^{-2}$ M | BuCh $2.9 \cdot 10^{-3}$ M | |
| Liver | | | | | | |
| 1 ♂ | 3.3 | 2.6 | 1,820 | — | 5,425 | |
| 2 ♂ | 1.9 | 1.4 | 1,050 | — | 3,115 | |
| 3 ♀ | 2.1 | 1.7 | 2,275 | — | 6,335 | |
| 4 ♀ | 2.8 | 2.3 | 2,695 | — | 7,385 | |
| 5 ♂ | 3.2 | 2.6 | 1,260 | — | 3,605 | |
| | 2.7 ± 0.23 n = 5 | 2.1 ± 0.20 n = 5 | 1,820±306 n = 5 | — | 5,173±806 n = 5 | |
| Gastric mucosa | | | | | | |
| 1 ♂ | 3.3 | 2.6 | 3,220 | — | 8,435 | |
| 2 ♂ | 1.9 | 1.4 | 2,870 | — | 5,880 | |
| 3 ♀ | 2.1 | 1.7 | 1,750 | 560 | 3,220 | |
| 4 ♀ | 2.8 | 2,3 | 2,590 | 875 | 3,780 | |
| 5 ♂ | 3.2 | 2.6 | 2,170 | 525 | 3,710 | |
| 6 ♀ | 2.7 | 2.1 | 1,870 | 525 | 2,870 | |
| | 2.7 ± 0.23 n = 6 | 2.1 ± 0.20 n = 6 | 2,403±241 n = 6 | 621 ± 85 n = 4 | 4,649±870 n = 6 | |
| Intestinal mucosa | | | | | | |
| 1 ♂ | 3.3 | 2.6 | 460 | 190 | 400 | 0.48 |
| 2 ♂ | 1.9 | 1.4 | 290 | 93 | 176 | 0.53 |
| 3 ♀ | 2.1 | 1.7 | 270 | 70 | 80 | 0.88 |
| 4 ♀ | 2.8 | 2.3 | 618 | 245 | 408 | 0.60 |
| 5 ♂ | 3.2 | 2.6 | 548 | 163 | 350 | 0.47 |
| 6 ♀ | 2.7 | 2.1 | 525 | 257 | 327 | 0.79 |
| | 2.7 ± 0.23 n = 6 | 2.1 ± 0.20 n = 6 | 452±58 n = 6 | 170±31 n = 6 | 290±54 n = 6 | 0.63±0.07 n = 6 |

type of enzyme. — TORDA and WOLFF (1952) investigated the acetylcholine synthetizing and hydrolyzing enzymes in the rat's brain and recorded that hypophysectomy but not adrenalectomy is followed by a reduction in cholinacetylase only and that this change can be restored by prolonged administration of ACTH in vivo.

*Submaxillary gland.* This series comprises 8 cats examined 1 to 5 days after removal of both adrenals. As described in the pre-

Table 8.

**Table 8.**

*Adrenalectomized cats treated with DOCA. Cholinesterase activities in homogenates and extracts of salivary gland as $b_{30}$ per g tissue and (in brackets) in the whole gland. The per cent figures denote activity in extract as compared with homogenate. This group of cats, with their individual numbers, is identical with the corresponding group referred to in the preceding paper.*

| No. and sex | Days after adr.ect. | Activity | | | | | | MCh/BuCh |
|---|---|---|---|---|---|---|---|---|
| | | ACh $3.3 \cdot 10^{-3}$ M | | MCh $3.1 \cdot 10^{-3}$ M | | BuCh $2.9 \cdot 10^{-3}$ M | | |
| 1. Homog. ..... | 4 | 4,270 (4,398) | | 2,170 (2,235) | | 1,470 (1,514) | | 1.48 |
| ♀ Extract ..... | | 1,353 (1,394) | 32 % | 653 (673) | 30 % | 443 (456) | 30 % | 1.47 |
| 2. Homog. ..... | 6 | 5,110 (4,497) | | 2,639 (2,322) | | 910 (801) | | 2.90 |
| ♀ Extract ..... | | 1,703 (1,499) | 33 % | 653 (575) | 25 % | 373 (328) | 41 % | 1.75 |
| 3. Homog. ..... | 6 | 3,990 (3,192) | | 1,820 (1,456) | | 910 (728) | | 2.00 |
| ♀ Extract ..... | | 1,003 (802) | 25 % | 303 (242) | 17 % | 210 (168) | 23 % | 1.44 |
| 4. Homog. ..... | 6 | 4,480 (3,270) | | 2,660 (1,942) | | 840 (613) | | 3.17 |
| ♀ Extract ..... | | 1,237 (903) | 28 % | 583 (426) | 22 % | 327 (239) | 39 % | 1.78 |
| 5. Homog. ..... | 6 | 4,690 (3,424) | | 2,030 (1,482) | | 700 (511) | | 2.90 |
| ♀ Extract ..... | | 1,470 (1,073) | 31 % | 560 (409) | 28 % | 537 (392) | 77 % | 1.04 |
| 6. Homog. ..... | 7 | 4,130 (2,891) | | 1,610 (1,288) | | 770 (539) | | 2.09 |
| ♀ Extract ..... | | 1,353 (947) | 33 % | 420 (294) | 26 % | 397 (278) | 52 % | 1.06 |
| 7. Homog. ..... | 7 | 3,990 (3,751) | | 1,610 (1,513) | | 980 (921) | | 1.64 |
| ♂ Extract ..... | | 1,563 (1,469) | 39 % | 653 (614) | 41 % | 397 (373) | 41 % | 1.64 |
| Homog. ..... | | $4,380 \pm 156$ ($3,632 \pm 232$) | | $2,077 \pm 167$ ($1,748 \pm 156$) | | $940 \pm 95$ ($804 \pm 131$) | | 2.31 $\pm$ 0.25 |
| Extract ..... | | $1,383 \pm 27$ ($1,155 \pm 35$) n = 7 | 32 % $\pm$ 1.7 | $546 \pm 51$ ($462 \pm 62$) n = 7 | 27 % $\pm$ 2.8 | $383 \pm 38$ ($319 \pm 37$) n = 7 | 43 % $\pm$ 6.6 | 1.45 $\pm$ 0.04 n = 7 |

ceding paper the salivary gland undergoes atrophy after this operation, the weight of the gland in some instances being only half the weight of normal controls. With daily injections of saline there was only a slight dehydration of the gland. The esterase activities per gram tissue are increased after adrenalectomy. In the group of 8 cats estimations on the homogenate reveal an increase as compared with the controls of 42 % ($P < 0.001$), 46 % ($P < 0.01$) and 50 % ($0.05 < P < 0.1$), respectively, for the three substrates ACh, MCh and BuCh. Because of the great deviations from the mean the increase in activity towards BuCh is less significant. Estimations on the corresponding extracts show an increase of 62 %, 60 % and 105 %, respectively, indicating such changes in the gland after adrenalectomy that the esterases more easily are brought into solution and, further, that after adrenalectomy the "pseudo" type is even more easily extractable than the "true" type. — The values for the whole gland, in the table given in brackets, are less elevated, obviously because of the atrophy. — From Table 6 it will be seen that after adrenalectomy the esterase activity of the salivary gland increases rapidly; the change is distinct at the first day after operation.

*Gastro-intestinal mucosa and liver.* The results are summarized in Tables 2, 3 and 4. In the liver and gastric mucosa adrenalectomy does not cause a detectable change in the pattern of cholinesterase content. In the mucosa of the small intestine the esterase activity is considerably higher than in the normal group. Chiefly the "true" type is increased. With the extract the increase towards ACh is 49 % ($P < 0.05$) above normal.

**Cats on reduced food.**

In our previous studies on histaminase and on the atrophy of the gastro-intestinal mucosa a series of cats maintained on reduced food was included. These cats lost about 20 % in body weight during the course of 4 weeks. Six of these cats are referred to in the preceding paper. Since after the operation of hypophysectomy or adrenalectomy a few cats lost in body weight it was decided to examine cats on reduced food also for cholinesterases. Such examination, moreover, seemed pertinent in view of the fact that both the "pseudo" and the "true" cholinesterase activities have been found to fall in human undernutrition (HUTCHINSON, MCCANCE and WIDDOWSON 1951). HARRISON and BROWN (1951) noted that the cholinesterase activity in the liver and serum of female rats,

but not of males, is lowered in undernutrition. WATERLOW (1950) found that in undernourished infants the cholinesterases in the liver and serum increased as the state of nutrition improved.

Observations in undernourished cats are summarized in Table 7. In the intestinal mucosa the esterase activities per gram tissue are reduced. Compared with normal controls the reduction against the three substrates amounts to 24 %, 28 % and 13 %, respectively. In the salivary gland, gastric mucosa and liver no changes are detectable.

*Sex differences.* No such differences were noted in the levels of cholinesterase content either in the normal controls or the groups subjected to hypophysectomy, adrenalectomy or reduced food.

### Effect of DOCA in Adrenalectomized Cats.

*Submaxillary gland.* The cats were given daily doses of 0.5—1.0 mg/kg intramuscularly. In homogenates as well as extracts the mean values of esterase activities are lower (Table 8) than in the untreated adrenalectomized group (Table 6). The standard error of the mean being large, the difference between the two groups is not significant when evaluated by Student's t-test. — Experiments with larger doses of DOCA seem justified.

*Intestinal mucosa.* In three of these cats the activity of homogenates and extracts towards ACh was determined. Two cats were examined at the sixth day of operation and treatment, the third at the seventh day. The means were 1,218 in the homogenates and 1,085 in the extracts, thus, there is no significant difference as compared with the untreated adrenalectomized cats of Table 4.

## Discussion.

The conspicuous increase in cholinesterase activity of the salivary gland after hypophysectomy or adrenalectomy and in the intestinal mucosa after adrenalectomy warrants consideration. The rapidity of the change directs attention towards other mechanisms than an acceleration in the synthesis of the enzyme protein. Some enzyme activator(s) or the elimination of specific inhibitors may be instrumental. Since corresponding changes could not be demonstrated in other tissues rich in cholinesterase the factors responsible for enzyme activation seem likely to be confined to the salivary gland and intestinal mucosa. It is tempting to assume that in the salivary gland the increase in cholinesterase activity is

linked to some yet unknown function of the gland under pituitary-adrenal control, as discussed in the preceding paper.

There are few references, so far, to experimental procedures inducing a rise in vivo in the level of enzymes related to "local hormones". TORDA and WOLFF (1952) recorded that administration of ACTH in rats increased the ability of brain homogenate to synthesize acetylcholine. It has been claimed that in rabbits injections of progesterone or gonadotropic hormones cause up to a several hundred times increase in the histaminase concentration of the endometrium (SWANBERG 1950, AHLMARK and SWANBERG 1953). These reports, however, are not in accordance with more recent studies of ROBERTS and ROBSON (1953) working mainly on rats. In particular, they found no increase in the uterus by treatment with progesterone. In preliminary experiments on the rabbit they also failed to confirm the claim that the concentration of endometrial histaminase is greatly increased by progesterone treatment. In the parotid gland of the cat STRÖMBLAD (1956) found that sympathetic denervation is followed by an increase of about 20 % in the concentration of amine oxidase.

In conclusion, it might be emphasized that determinations of the over-all ratio of the relative activities of the two types of esterase in homogenates as in the present study not necessarily reflect local changes in enzyme concentration at definite sites within the tissue or organ. Histochemical procedures, again, are not yet well adapted to quantitative determinations (for discussion see KOELLE 1950, 1951).

## Summary.

1. The cholinesterase activity of the "true" and "pseudo" types of homogenized submaxillary gland, gastro-intestinal mucosa and liver was determined in five groups of cats: 1) normal, 2) hypophysectomized, 3) adrenalectomized, 4) adrenalectomized maintained on DOCA, 5) undernourished.

2. In normal cats the cholinesterase of the submaxillary gland is predominantly of the "true" type, in the liver the "pseudo" type only could be found, in the gastric mucosa the "pseudo" type prevails beside merely detectable amounts of "true" esterase, while the mucosa of the small intestine is abundant in both types of esterase.

3. Hypophysectomy and adrenalectomy are followed by a considerable increase in the concentrations of "true" and "pseudo"

cholinesterase in the salivary gland. Similar changes occur in the intestinal mucosa after adrenalectomy, while they are absent in the liver and gastric mucosa.

4. In adrenalectomized cats treatment with DOCA possibly had a slight (statistically not significant) effect in lowering the cholinesterase levels of the salivary gland towards normal, while no such effect was observed in the intestinal mucosa.

5. In cats on reduced food a decrease in both types of esterase activity was found in the mucosa of the small intestine, whereas in the salivary gland, gastric mucosa and liver no changes were detectable.

We are grateful to Dr. DORA JACOBSOHN, who performed the operation of hypophysectomy and to Dr. K. B. AUGUSTINSSON for advice and criticism.

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# The Effect of a Vasodilator (Priscol) on the Gastric Secretion.

By

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Ever since POPIELSKI in 1920 discovered that histamine was an effective stimulus for gastric secretion, numerous investigators have used this drug for studies in gastric physiology. Although many other substances are gastric secretagogues, none has been discovered that compares with histamine in effectiveness. From investigations by IVY and FARREL (1925), KLEIN and ARNHEIM (1932), GROSSMAN and IVY (1946) it is known that histamine acts directly on the mucosal cells and not through nervous pathways.

As it is well known that histamine is a potent vasodilator, and that there is a close relationship between the rate of secretion and the vascularisation of the mucosa (WOLF and WOLFF 1943, VANE and THOMPSON 1952) one would expect that any substance that acts as a vasodilator should have a similar effect as histamine on the gastric secretion. Papers have appeared that indicate that such an effect is found for some commonly used vasodilators, *e. g.* benzazolinchloride (Priscol Ciba). THIELE and SCHÜMANN (1942) found an effect in cats almost similar to that of histamine. SCHNETZ and FLUCH (1941) similarly found an effect in human subjects. For a review of the literature on the effect of Priscol on gastric secretion the reader is referred to ROCH-BESSER (1950).

From the papers that have appeared it is not possible to compare accurately the effects of Priscol with that of histamine, and this is the intention of the present paper. In order to do this it is necessary to find out the normal dose-effect relationship

for histamine on the gastric secretion and to compare it with the corresponding dose-effect curve of Priscol. Due to the rapidity with which histamine is destroyed in the blood stream it is necessary to study its dose-effect curve when it is injected continuously by the route. ÖBRINK (1948) described the relationship for Heidenhain-pouch dogs as

$$v = a(1 - e^{-kr}) \quad (1)$$

where $v$ is the volume-secretion rate in ml/min, $a$ the maximal secretion rate (ml/min), $r$ the amount of histamine injected per unit of time (mg/hour) and $k$ a constant.

The dose-effect curve for Priscol is not known and therefore we attempted to determine this in Heidenhain-pouch dogs. A comparison of the two dose-effect curves should be made on an equimolar basis.[1]

## Methods.

The experiments were performed on a Heidenhain-pouch dog. Before every experiment the dog was starved for at least twelve hours and only when there was no basal secretion was the dog used for these experiments. Histamine-dihydrochloride was given as a continuous intravenous injection by a special injection device (ÖBRINK 1948). When a steady state of the secretion rate was obtained, Priscol was injected also by the continuous intravenous route and the two injections were continued for the rest of the experiment. The acidity was titrated on 0.1 ml sample of juice from the pouch using bromthymol-blue as an indicator.

## Results.

It was not possible to obtain a dose-effect curve by using Priscol as stimulating agent, because Priscol alone in moderate doses gave no secretion at all. In comparison it may be noted that while a dose of 0.272 μmols/kg/hour of histamine gave a secretion rate of 50 % of the maximal one, as much as 1.04 μmols/kg/hour of Priscol did not have any measurable effect on the secretion. Only when such high doses were given that the animal became disturbed was a small quantity of secretion obtained. 3.24 μmols/kg/hour evoked a secretion of 0.05 ml/min or about 5 % of the maximal secretion rate ($a$ in eq. 1). These results therefore seem to disagree with previous authors, who found Priscol to be a potent stimulator.

[1] The molecular weight of histamine-dihydrochloride is 184.15 and of Priscol 196.7.

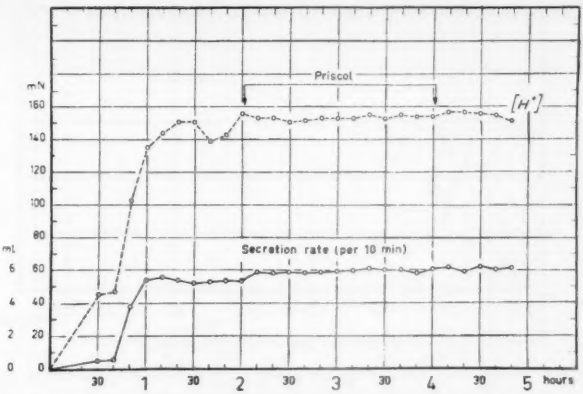


Fig. 1. *A typical secretion experimen'*. During the whole experiment histamine was given intravenously (0.89 mg/hour). Between 2nd and 4th hours Priscol was also given intravenously (1.77 mg/hour). Increment in secretion rate = 0.7 ml/10 min.

If on the other hand Priscol was given continuously to the animal when it was already secreting gastric juice in response to the continuous intravenous injection of histamine a significant increase in the rate of secretion was observed. It was therefore concluded that although Priscol *per se* had no stimulating effect it could potentiate the histamine stimulation. A typical experiment showing this is seen in figure 1 where histamine was given during the whole experiment and a Priscol injection started when a steady state of the secretion was already established. After a very short time after the start of the Priscol injection a new higher level of the secretion rate was obtained. In this particular experiment it can also be seen that after interrupting the injection of Priscol its effect remains for at least one hour. This is contrary to what happens when the injection of histamine is stopped. In that case the secretion rate drops to zero within 20 to 30 minutes.

## Discussion.

It was found that moderate doses of Priscol alone had no stimulating effect on the gastric secretion unless histamine was injected simultaneously. In that case it potentiated the effect of the histamine. This potentiation apparently varied according to the concomitant dose of histamine. To obtain a value for the effect

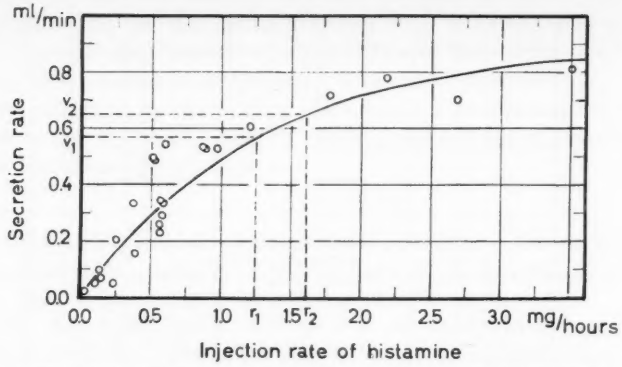


Fig. 2. *The relationship between injection rate of histamine-dihydrochloride* ($r$) *and secretion rate* ($v$). The continuous line represents the equation $v = 9.15\,(1 - e^{-0.77r})$.

of Priscol we have introduced a "coefficient of histamine equivalence" $= f$. This coefficient was obtained in the following way. First the normal relationship between the dose of histamine and its corresponding secretion rate was experimentally established. From the experimental data the best fit of the formula:

$$v = a(1 - e^{-kr})$$

was calculated by the method of least squares (fig. 2). If in an experiment, the secretion rate due to a histamine injection was $v_1$ ml/min and the Priscol in a dose of $p$ mg/hour caused an increment of the secretion rate to $v_2$ ml/min this same increment in secretion rate could have been obtained if the injection rate of histamine had been increased from $r_1$ to $r_2$ mg/hour. The quotient $(r_2 - r_1)/p$ expresses the histamine equivalence per unit of Priscol dose. When this quotient is calculated with both agents expressed in mol/hour we get our coefficient $f$. This method of calculating implies a straight line dose-effect relationship for Priscol for a certain constant histamine stimulation. This is certainly not the case, but $p$ has been kept within very narrow limits thus permitting this approximation. Figures 3 and 4 show the relation between $f$ and the secretion rate $v_1$ and the injection rate of histamine $r_1$ respectively. It is obvious that the effect of Priscol is increasing for increasing initial stimulation with histamine. At the highest preceding histamine stimulation the effect of Priscol is just as potent as histamine itself while without histamine it had no effect whatsoever.

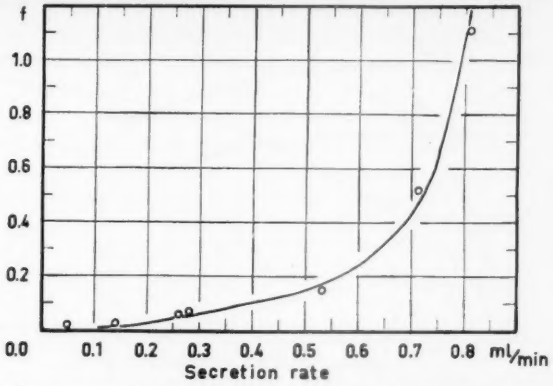


Fig. 3. *The relation between the "coefficient of histamine equivalence", f, and the control secretion rate.* The continuous line was constructed from eq. (8), where $k = 0.77$, $a_1 = 9.15$ and $a_2 = 9.70$.

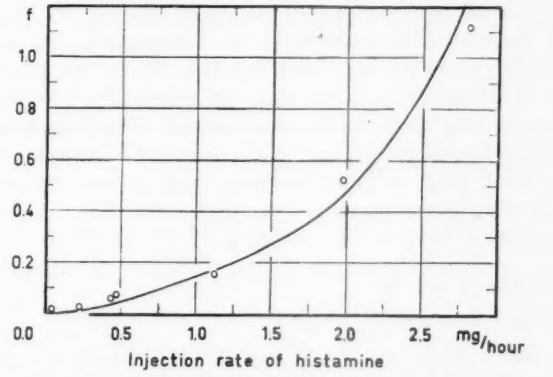


Fig. 4. *The relation between the "coefficient of histamine equivalence", f, and the injection rate of histamine.* The continuous line was constructed from eq. (9) with the constants $k_1$, $a_1$ and $a_2$ as in fig. 3.

From these results it seems probable that the actions of histamine and Priscol are not identical. Although histamine is considered to be a potent vasodilator its effect on the gastric secretion cannot be accounted for by that property (at least not entirely). Its effect on the secreting cells must be ascribed to some other property. It is of considerable interest to notice that vasodilation in itself is not sufficient to induce gastric secretion.

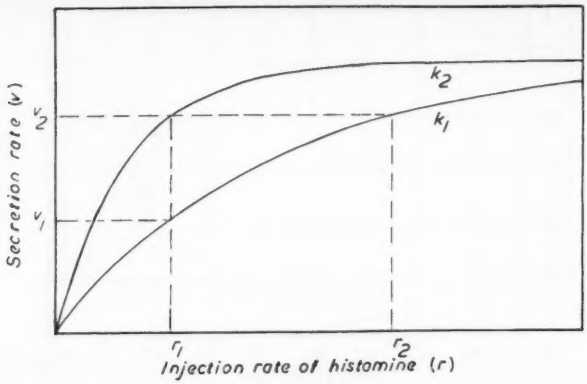


Fig. 5. *Dose-effect curves* representing two different $k$-values in $v = a(1 - e^{-kr})$.

It may be of interest to speculate whether the vasodilation causes an increased *sensitivity* to histamine, *i. e.* an increment in the constant $k$ in formula (1), or if it increases the *maximal secretory response*, *i. e.* an increment in $a$. These two possibilities could be tested if the coefficient $f$ in relation to $v_1$ or $r_1$ could be theoretically derived from the formula (1) and compared to the experimental results in figures 3 and 4. According to the definition $f$ is $(r_2 - r_1)/p$. Assuming that $p$ remains constant, $f$ is directly proportional to $(r_2 - r_1)$. Thus we can calculate $(r_2 - r_1)$ as a function of $v_1$ or $r_1$.

*1. Change in k (fig. 5):*

Solving $r$ from eq. (1) we get

$$r = -\frac{1}{k} ln\left(1 - \frac{v}{a}\right) \quad (2)$$

From fig. 5 it is obvious that

$$r_1 = -\frac{1}{k_1} ln\left(1 - \frac{v_1}{a}\right) = -\frac{1}{k_2} ln\left(1 - \frac{v_2}{a}\right) \quad (3)$$

$$r_2 = -\frac{1}{k_1} ln\left(1 - \frac{v_2}{a}\right) \quad (4)$$

Solution of (3) and (4) gives

$$r_2 - r_1 = \left(\frac{k_2}{k_1} - 1\right) \cdot r_1 = constant \cdot r_1 \quad (5)$$

This means that the $f$-values should form a straight line through the origin when plotted against $r_1$. A comparison with fig. 4 reveals

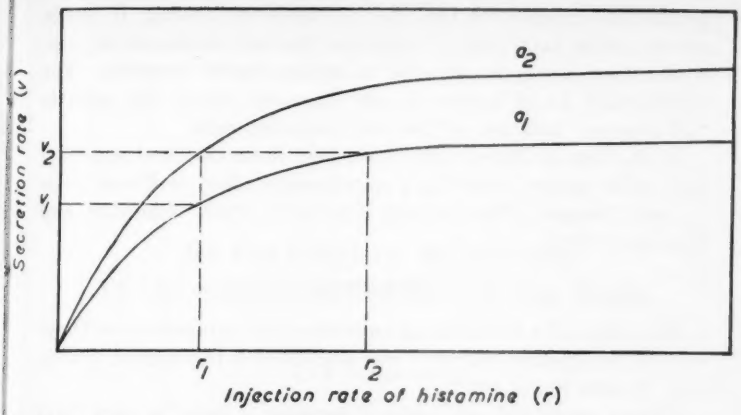


Fig. 6. *Dose-effect curves* representing two different $a$-values in $v = a(1 - e^{-kr})$.

a profound discrepancy between theory and results. Thus it seems very unlikely that the effect of Priscol is a sensitization of gastric glands towards histamine.

*2. Change in a (fig. 6):*

Starting again from eq. (2) and using fig. 6, we get

$$r_1 = -\frac{1}{k} ln\left(1 - \frac{v_1}{a_1}\right) = -\frac{1}{k} ln\left(1 - \frac{v_2}{a_2}\right) \quad (6)$$

$$r_2 = -\frac{1}{k} ln\left(1 - \frac{v_2}{a_1}\right) \quad (7)$$

Combination of (6) and (7) gives

$$r_2 - r_1 = -\frac{1}{k} ln \frac{a_1^2 - a_2 v_1}{a_1^2 - a_1 v_1} \quad (8)$$

This relation is represented as the solid line in fig. 3. When (1) is used for substitution of $v_1$ in (8) this changes to

$$r_2 - r_1 = -\frac{1}{k} ln \frac{a_1 - a_2(1 - e^{-kr_1})}{a_1 e^{-kr_1}} \quad (9)$$

This relation is given by the line in fig. 4.

From these results it seems possible to explain the effect of Priscol as being mainly due to an increment in the maximal secretory rate which histamine alone can produce.

Our results may indicate that the state of vascularisation in the stomach is an important factor in determining the maximal volume output for a given type of stimulation. More direct ex-

perimental evidence for this idea is, however, lacking. It seems on the other hand safe to conclude that an increment in vascularisation *per se* is not able to induce gastric secretion. The results may be of interest in the discussion about the possible "all-or-none" reaction of the acid secreting cells.

In the case of Priscol, only very high doses initiated secretion and earlier papers reporting a secretagogue effect of Priscol refer to such dosages (THIELE and SCHÜMANN 1942, EMMELIN and KAHLSON 1944).

## Summary.

The effect of a vasodilating substance benzazolinchloride (Priscol) on the gastric secretion was studied in a Heidenhain pouch-dog. It was found that:

Priscol alone did not initiate secretion except in very high doses, but that it could enhance secretion already induced by histamine. This potentiating effect could be related to the strength of the existing stimulation.

The higher the preceding secretion rate, the higher the effect of Priscol expressed as a coefficient of histamine equivalence.

It was suggested that the action of histamine on the gastric glands is not due to its vasodilating property. The probability that the vasodilation causes an increased maximal secretory capacity was discussed.

The experiments were defrayed by grants from the Medical Faculty of Uppsala.

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# On the Sulphate Metabolism of the Sulphocerebrosides of the Brain.

By

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Protagon, the starting material for the isolation of cerebrosides, was first isolated by COURBE (1834) and by FREMY (1841). Its name derives from LIEBREICH (1865), who used it to denote substances soluble in hot ethanol but insoluble in cold. Protagon has later been isolated from a number of organs, *e. g.* the brain, lung, red and white blood corpuscles, kidneys and adrenals. The fact that it is not a homogeneous substance was demonstrated in 1900 by WÖRNER and THIERFELDER.

THUDICHUM isolated the cerebrosides phrenosin and kerasin from the brain, and elucidated their chemical composition. He also showed that protagon contains sulphur. KOSSEL and FREITAG, LESERN and GIES, and CRAMER found only 0.5—0.8 per cent of sulphur, and RUPPEL (1895) extremely little or none. KOCH (1910) demonstrated that the ester sulphate is bound to the carbohydrate in protagon. He considered it to be a phosphatide cerebroside sulphatide. LEVENE (1912) isolated a sulpholipid containing 2.66 per cent of sulphur.

Studies of the protagon of the human brain were made by BLIX (1933), who found it to contain 1.46 per cent of $H_2SO_4$. He subsequently isolated a sulpholipid with an $H_2SO_4$ content of 9.89 per cent. He stated that 20 to 25 per cent of the cerebrosides are esterified with sulphuric acid.

DZIEWIATKOWSKI (1945) administered sodium sulphide containing $S^{35}$ orally to rats and followed its uptake in the urine,

faeces and various tissues. He recovered 0.02 per cent of the dose given in the brain. Similar results were obtained by KULWICH, PEARSON and LANKENAU (1954) in the rabbit.

BOSTRÖM and ODEBLAD (1953) studied the uptake of sulphate in the central nervous system of the adult rat by means of autoradiography. In the cerebrum, the cerebellum and the spinal cord, the grey matter showed the greatest fixation. In the cerebellum, a particularly high uptake was noted in the granular layers of the cortex. In the opinion of the authors, it is not improbable that the main incorporation of sulphate takes place in the sulpholipids, in which the sulphate is bound to an alcohol group in an ester linkage, as in the sulpho-mucopolysaccharides.

FEYRTER, PISCHINGER and LIEB (1942) investigated the presence of chromotropic lipids. They found that, in Gaucher's disease, the Gaucher cells stained metachromatically. When the cerebrosides were isolated, the pure substance also exhibited metachromasia.

The so-called Reich $\pi$-granules in Schwann's cells in the peripheral nerves of human subjects were studied by CIACCIO (1951) and by NOBACH (1953), they found metachromatic substances in these cells. According to NOBACH, this property is to be ascribed to the presence of a phosphatide or a sulphatide.

The sulpholipid was isolated by BOOIJ, DEIERKAUF and HEGNAUR-VOGELENZANG (1953), using the method of BLIX (1933). The sulphocerebroside was found to stain metachromatically with toluidine blue.

## Experimental.

On the basis of the aforegoing data, my object was to study the exchange of the sulphate groups in the cerebrosides by means of $S^{35}$. BLIX's method (1933) was used for isolation of the sulpholipid, but it was not possible to perform the last stage, since the basic material was insufficient. The radioactivity of the different preparations was determined in a surface of 1.58 $cm^2$ and a layer of infinite thickness. At least 10,000 counts were made. The radioactivity of the background was measured for 30 minutes before each determination.

Thirteen rats, weighing about 250 g, were given 2 $\mu$C of $S^{35}$-labelled sodium sulphate per g of body weight by subcutaneous injection. The animals were sacrificed in two groups, 52 and 256 hours later, respectively. The brains were dissected out and rinsed

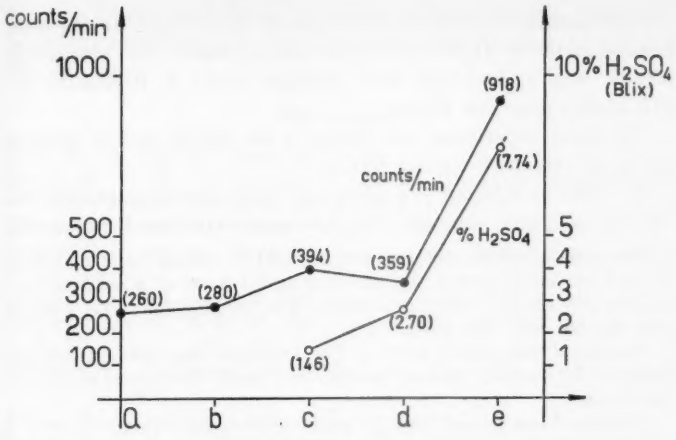


Fig. 1.

a = after acetone extraction.
b = after ether extraction.
c = alcohol extract through Dowex columne.
d = after pyridine fractionating.
e = after acetic acid fractionating.

in distilled water. Total weight: 22.2 g. They were then treated with acetone for 24 hours. The solvent was changed 6 times, using 200 ml each time. The temperature was 0° C throughout. The solution was filtered and the residue dried *in vacuo*. Weight: 4.06 g. Radioactivity: 260 counts/min.

This fraction was suspended in ether and was then extracted for 3—4 hours. The solvent was changed 3 times, altogether 600 ml. Temperature: 0° C. Filtration and drying of the residue followed. Weight: 1.85 g. Radioactivity: 280 counts/min.

This fraction was boiled in 99.5 per cent ethanol for 5 minutes. The hot solution was passed through an anion exchange column (Dowex 2) 250—500 mesh 1 cm in diameter and 20 cm in length, at 42° C. It was then cooled to 0° C, when a white precipitate of protagon was formed. This was suctioned off, washed in ether and dried. Weight: 0.353 g. Radioactivity: 394 counts/min.

The dried precipitate was dissolved in pyridine during slow heating to 60° C. It was cooled to 0° C and placed in the refrigerator overnight. The precipitate then formed was removed by suction, washed in ether and dried. Weight: 0.138 g. Radioactivity: 359 counts/min.

This fraction was dissolved in glacial acetic acid during slow heating to 60° C. It was cooled in running water. The precipitate formed was washed and dried. Weight: 0.010 g. Radioactivity: 918 counts/min. See Figure 1.

The same experiment was made in the rabbit and in another group of rats with similar results.

In order to rule out the possibility that the radioactivity was due to inorganic sulphate, the following experiment was made.

One gram of cattle protagon was brought to boiling point in 100 ml of 99.5 per cent ethanol. To this was added 1 ml of a solution containing $S^{35}$-labelled sodium sulphate. The protagon-sulphate solution was divided into two parts.

*Fraction 1* was cooled to 0° C. The protagon then precipitated was removed by suction, washed in ether and dried. Radioactivity: 28,811 counts/min.

*Fraction 2* was passed through an anion exchange column (Dowex 2) at 42° C. It was cooled and then treated as fraction 1. Radioactivity: 0 counts/min.

The same experiment was made with cattle protagon and chondroitin sulphuric acid containing $S^{35}$. This acid is known to occur in the walls of the blood vessels. After the hot solution had been passed through the anion exchange column as described above in the procedure of preparation, the radioactivity was found to be 0. The characteristic rise in the activity after fractionation in glacial acetic acid was also lacking. Evidently the chondroitin sulphuric acid had been removed during the course of the isolation of the sulphocerebroside.

## Discussion.

It has been shown by DZIEWIATKOWSKI (1951) and by BOSTRÖM and ODEBLAD (1952), among others, that the greatest incorporation of sulphate occurs in those tissues having a high sulphomucopolysaccharide content. In cartilage, the highest uptake takes place in the chondroitin sulphuric acid.

In all the experiments, fractionation with glacial acetic acid resulted in a marked rise in the radioactivity of the protagon, analogous to the rise in the sulphur content. Consequently, it is evident that some exchange takes place between the sulphate groups in the sulphocerebrosides of the brain and the radioactive free sulphates.

## Summary.

$S^{35}$-labelled sodium sulphate is injected subcutaneously into rats, and the radioactivity in the cerebroside fractions of the

brain is determined. Some exchange is found to take place between the ester sulphate of the sulphocerebrosides and the labelled free sulphates.

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# Mammary Gland Growth in Hypophysectomized Rats Injected with Ovarian Hormones and Insulin.

By

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The development and growth of the mammary gland promoted by ovarian hormones in intact animals is inhibited after hypophysectomy (FOLLEY and MALPRESS 1948, JACOBSOHN 1954). Mammary gland growth can be obtained in hypophysectomized animals after administration of anterior pituitary extracts, either alone or together with ovarian hormones. Much work has been done to elucidate the nature of the anterior hypophysial factor(s) stimulating the mammary gland tissue and/or making it responsive to the action of ovarian hormones. The observations made on hypophysectomized rats strongly suggest prolactin and growth hormone to be the hypophysial factors necessary, in addition to gonadal hormones, for the development and growth of the mammary gland (LYONS 1951, LYONS, JOHNSON, COLE and LI 1955). From the evidence available it may be assumed that prolactin is acting directly on the mammary gland tissue. The mode of action of growth hormone is so far conjectural (for lit. see FOLLEY and MALPRESS 1948, FOLLEY 1952, COWIE and FOLLEY 1955, LYONS et al. 1955).

In recent years little attention has been paid to the part played by the depression of metabolic functions for the failure of growth processes to occur in the mammary gland of animals supplied with ovarian hormones but deprived of their pituitary gland. Growth hormone and presumably prolactin can elicit profound

changes in the intermediary metabolism (ASTWOOD 1955, FOÀ, GALANSINO, WEINSTEIN and MAGILL, 1955). The increased responsiveness of the mammary gland tissues after administration of these hormones to hypophysectomized animals may reasonably be assumed to be due in part to alterations of the general metabolism and, possibly, to a release of other hormones with metabolic actions.

SALTER and BEST (1953) and LAWRENCE, SALTER and BEST (1954) showed 1) that hypophysectomized rats tolerate daily injections of long-acting insulin during fairly long periods and 2) that the insulin administered to such rats promotes increased voluntary food intake, and changes in growth processes and the internal metabolism as judged from *e. g.* increased weight and length of the body, widening of the epiphysial disk of the tibia and increased nitrogen retention. In the present work the capability of the mammary gland to develop and grow has been investigated under conditions similar to those studied by SALTER and BEST. The experiments described below were performed on castrated, hypophysectomized female rats injected with oestrone, progesterone and long-acting insulin.

## Experimental.

Female rats from the colony kept at our Institute were used. Over 20 generations of albino rats had been thoroughly inbred by brother sister matings. Because of a high mortality rate occurring lately among the offsprings, this strain had recently been outbred by allowing the females to mate with males from a closed colony of piebald rats kept at the Department of Pharmacology. Besides these males no outside animals had been introduced into our stock. The diet consisted of bread dog biscuits, mixed grain and fresh milk daily, supplemented by raw meat once a week. During the period of injections glucose was added to the milk at about 21.00 hr. and, if necessary, additional bread and grain were then given to the animals. All rats were allowed to feed ad libitum.

The mammary glands were studied in groups of rats subjected to the following procedures:

*Group I:* (10 rats) Castration and injections of oestrone and progesterone.

*Group II:* (9 rats) Castration, hypophysectomy and injections of oestrone and progesterone.

*Group III:* (18 rats) As group II, but in addition daily injections of long-acting insulin: a) protamine zinc insulin, Vitrum and b) zinc insulin lente, Novo.

*Group IV:* (15 rats) As group III, but instead of insulin control solutions were injected a) a zinc protamine suspension (Vitrum) and b) a zinc solution ("lente minus insulin", Novo).

*Group V:* (13 rats) Castration and hypophysectomy and daily injections of a) protamine zinc insulin, Vitrum and b) zinc insulin lente, Novo.

As far as possible experiments on the different groups were done concomitantly and littermates were distributed equally among the various groups.

In addition to the groups just mentioned similar experiments have been done with local application of oestrone to three mammary glands on one side.

The ovaries were usually removed before the vaginal orifice opened, most of the rats being 3 to 4 weeks old at castration.

Hypophysectomy was performed by the parapharyngeal route at 10 to 51 days after castration, in the majority of the experiments at about one month after castration. The completeness of the removal of the pituitary gland was controlled by means of serial sections through the hypophysial capsule and adjacent tissues including the pituitary stalk and the median eminence. Experiments in which the microscopic examination revealed groups of cells within or outside the hypophysial capsule in addition to the pars tuberalis surrounding the pituitary stalk have been discarded.

Oestrone in a dose of 20 $\mu$g was injected intramuscularly every other day using a solution of 1,000 $\mu$g per ml arachis oil. Progesterone was given every 6th day intramuscularly in a dose of 5 mg. The preparation was a microcrystalline suspension (Lutocyclin, microcrist. 50 mg per ml, Ciba).

For the treatment with insulin two different commercial preparations, both containing 40 i. u. per ml were employed: 1) protamine zinc insulin (Vitrum) and 2) zinc insulin lente (Novo).

The injections of oestrone and insulin were begun simultaneously. The first injection of progesterone was given on the day following the start of oestrone administrations. The beginning of treatment varied between 19 and 53 days after hypophysectomy. Generally, however, injections were instituted at 3 weeks after hypophysectomy. Increasing doses of insulin were given subcutaneously according to the following scheme until the animals died: 1, 2 and 4 i. u. daily for 2 days, respectively, then 8 and 16 i. u. daily for 6 days, respectively, and lastly 24 i. u. daily. As may be seen below, most of the rats died during the daily administration of 16 i. u. of insulin. The dose of insulin was administered by one daily injection made at about 12 o'clock in the morning. Hypoglycemic attacks were not observed in hypophysectomized rats receiving less than 8 i. u. of insulin daily, that is within the first six days. No attempt was made to relieve a hypoglycemia occurring during the administration of 8 i. u. or more. The control solutions, lacking insulin activity but prepared so as to match the insulin preparations with regard to the concentration of constituents other than insulin, were given in amounts equal to the insulin preparations: 0.025,

0.05 and 0.1 ml daily for 2 days each, followed by 0.2 and 0.4 ml for 6 days, respectively, and lastly by 0.6 ml, subcutaneously. All injections were made with a 1.0 ml tuberculin syringe using magnifying spectacles for amounts of less than 0.05 ml.

The effectiveness of the oestrone and progesterone injections was judged from the vaginal opening, from vaginal smears and, post mortem, from the appearance of the uterus. The body weight of the rats was recorded before each operative procedure and at least twice a week during the period of injections.

Before the start of injections one (usually the second) of the right thoracic mammary glands was extirpated. At autopsy, performed one day or less after the last injection, the corresponding left gland and the left abdominal mammary gland were dissected out. These as well as the control glands were stained with gallocyanin chromalum and mounted in toto. In groups I and III the right abdominal mammary gland was removed in addition at autopsy and fixed in Bouin's solution. Parts of this gland were embedded in paraffin wax, cut into sections 10 $\mu$ thick and stained with hematoxylin eosin. The growth and development of the mammary glands were assessed from the whole mount preparations studied under the microscope with magnifications of 20 and 80. Supplementary information about the presence of secretion and the formation of smallest ducts or acini was obtained from the 10 $\mu$ sections examined microscopically. Details about the techniques used in the present work and about the criteria adopted for the scoring of the development and growth of the mammary glands have been given by JACOBSOHN (1948). In order to simplify the presentation of the material and because the variations of the type and degree of the development of the mammary glands were small within one and the same group of experiments typical examples are given in figures 1—12. Entries concerning the development of each mammary gland studied are omitted in the tables.

The liver and the adrenal glands were dissected out post mortem and weighed. Frozen sections of the adrenal glands, 10 $\mu$ thick, and stained with colloidal sudan were examined microscopically.

## Results.

The observations made on the mammary glands are illustrated by the photographs on plates 1 and 2. The results obtained from groups I, II and V will be given briefly. Tables 1 to 4 contain data from the experiments of groups III and IV.

*Group I:* Ten rats castrated at an age of 3 to 5 weeks were given daily injections of oestrone and progesterone from 2 months after castration onwards during periods varying from 10 to 31 days. The mammary glands presented the well known growth processes combined with slight secretion. As may be seen from fig. 1, plate 1, the proliferation of ducts predominated. During the

**Table 1.**

(Group III a):

*Hypophysectomized castrated rats. Daily injections of protamine zinc insulin, Vitrum, and inj. of 20 µg oestrone every other day + 5 mg progesterone every 6th day.*

| Exp. | Body weight at | | | Period of injections days | Wt. of both adrenals mg | Wt. of liver in % of body wt. |
|---|---|---|---|---|---|---|
| | castr. g | hyp.ect. g | exam. g | | | |
| 1 | 45 | 140 | 160 | 12 | 16.0 | 5.00 |
| 2 | 45 | 165 | 225 | 15 | 6.8 | 4.89 |
| 3 | 45 | 160 | 170 | 15 | 12.9 | 4.71 |
| 4 | 45 | 130 | 160 | 15 | 10.1 | 5.63 |
| 5 | 35 | 145 | 180 | 16 | 12.6 | 5.28 |
| 6 | 50 | 145 | 175 | 16 | 15.5 | 4.57 |
| 7 | 45 | 140 | 190 | 17 | 13.9 | 4.08 |
| 8 | 50 | 145 | 190 | 17 | 12.2 | 3.16 |

treatment the body weight of these rats increased by an average of 15 %. The mean ± standard error of the mean for the weight of both adrenal glands was 56 ± 1.8 (10) mg. The corresponding values for the weight of the liver in g and in per cent of the body weight were 7.4 ± 0.52 (9) and 3.1 ± 0.13 (9), respectively.

*Group II:* This group comprises 9 rats castrated when 3 to 5 weeks old and hypophysectomized at 10 to 53 days later. 5 of the 9 rats were hypophysectomized about one month after castration. The treatment with oestrone and progesterone was instituted at 16 to 53 days after hypophysectomy and the injections were given during periods varying from 10 to 27 days. The observations made on the mammary glands confirmed those of previous investigators. Growth was absent or negligible (fig. 2, plate 1). The body weight remained unchanged in one rat, in the two youngest ones there was an increase and in the others it decreased. For the whole group an average decrease of 3.3 % was found during the treatment. The mean weight of both adrenal glands was 9.54 ± 0.93 (9) mg and that of the liver 4.57 ± 0.44 (7) g or 3.0 ± 0.11 per cent of body weight.

*Group III a:* (table 1). Eight rats castrated at 3 weeks of age and hypophysectomized 1 month thereafter were injected with oestrone, progesterone and protamine zinc insulin during periods of 12 to 17 days. The treatment was given as described under "experimental", and started at 3 weeks after hypophysectomy.

**Table 2.**

(Group III b):

*Hypophysectomized castrated rats. Daily injections of zinc insulin lente, Novo, and inj. of 20 μg oestrone every other day + 5 mg progesterone every 6th day.*

| Exp. | Body weight at | | | Period of injections days | Wt. of both adrenals mg | Wt. of liver in % of body wt. |
|---|---|---|---|---|---|---|
| | castr. g | hyp.ect.[1] g | exam. g | | | |
| 1 | 85 | 170 (140) | 155 | 8 | 11.7 | 4.8 |
| 2 | 85 | 195 (170) | 195 | 8 | 13.1 | 4.9 |
| 3 | 50 | 135 | 150 | 9 | 7.8 | 5.3 |
| 4 | 65 | 115 | 150 | 10 | 10.0 | — |
| 5 | 35 | 120 | 150 | 10 | 8.6 | 5.3 |
| 6 | 75 | 165 | 210 | 12 | 9.5 | 3.7 |
| 7 | 70 | 240 (210) | 235 | 14 | 9.2 | 5.1 |
| 8 | 55 | 185 | 250 | 15 | 15.0 | 4.4 |
| 9 | 80 | 85 | 160 | 16 | 8.0 | — |
| 10 | 80 | 90 | 170 | 20 | 9.0 | 5.3 |

[1] The figures in ( ) are giving the weight at the start of injections.

In striking contrast to group II the mammary glands of all rats receiving insulin presented a picture similar to that found in group I. As may be seen from figs. 3 and 4, plate 1, a marked proliferation of the side buds and end buds of the ducts occurred. Alveoli were found only occasionally. Microscopic examinations of 10 μ sections revealed a slight distension of the ducts with secretion. The degree of growth was less than in the rats of group I with an intact pituitary gland (compare figs. 1 and 4, plate 1).

During the treatment the body weight increased by up to 36 % (table 1, cols. 3 and 4). The mean weight of both adrenal glands was $12.5 \pm 1.05$ (8) mg or about 22 % of that found in group I. Microscopic examination revealed a considerable atrophy of the cortex of the adrenal glands. The difference between the adrenal weight of the present group and group II was not significant ($0.1 > P > 0.05$). The weight of the liver was considerably increased. The mean value of $4.66 \pm 0.27$ per cent of body weight is significantly higher than that found in the rats of group I with an intact pituitary gland ($P < 0.001$).

*Group III b:* (table 2). The treatment given to the 10 rats of this group was the same as in group III a except for the insulin

**Table 3.**

(Group IV a):

*Hypophysectomized castrated rats. Daily injections of protamine zinc suspension, Vitrum, and injections of 20 µg oestrone every other day + 5 mg progesterone every 6th day.*

| Exp. | Body weight at | | | Period of injections days | Wt. of both adrenals mg | Wt. of liver in % of body wt. |
|---|---|---|---|---|---|---|
| | castr. g | hyp.ect. g | exam. g | | | |
| 1 | 45 | 155 | 155 | 10 | 7.4 | 3.55 |
| 2 | 45 | 135 | 135 | 15 | 10.4 | 2.96 |
| 3 | 45 | 160 | 145 | 16 | 8.9 | 3.45 |
| 4 | 45 | 165 | 140 | 16 | 9.5 | 3.93 |
| 5 | 35 | 160 | 160 | 16 | 10.0 | 3.13 |
| 6 | 55 | 130 | 140 | 17 | 10.5 | 3.21 |
| 7 | 45 | 135 | 140 | 18 | 8.3 | 2.50 |
| 8 | 35 | 165 | 165 | 23 | 10.2 | 3.33 |

preparation used. The age at castration varied between 23 and 41 days. The hypophysectomy was performed 14, 20—22 (4 exp.), 36—38 (4 exp.) or 51 days later. Injections were started between 3 weeks and 2 months after hypophysectomy. Already 8 to 10 days after the beginning of treatment a marked proliferation of ducts was observed in the mammary glands (figs. 6 and 7, plate 1). The changes occurring in the mammary glands in this group of rats receiving zinc insulin lente were similar to those found in group III a treated with protamine zinc insulin. The degree of growth was, perhaps, somewhat less than in the foregoing group. From the beginning until the end of the injections the body weight increased, as in group III a, and the weight of the liver in per cent of body weight was higher than in the rats with intact pituitary gland (group I). The mean weight of both adrenal glands was $10.2 \pm 0.74$ (10) mg.

*Group IV a:* (table 3). This group of 8 rats was the control for group III a. Instead of protamine zinc insulin a suspension of protamine zinc was injected. The mammary glands examined 10 to 23 days after the first injection were similar to those of group II. Growth was absent or negligible (fig. 5, plate 1). The body weight remained practically unchanged during the period of treatment. The mean values for the weight of both adrenal glands, $9.4 \pm 0.39$ (8) mg, and for the weight of the liver in per cent of body weight, $3.26 \pm 0.15$, were not different from the corresponding values found in group II.

**Table IV.**

(Group IV b):

*Hypophysectomized castrated rats. Daily injections of lente minus insulin, Novo, and injections of 20 μg oestrone every other day + 5 mg progesterone every 6th day.*

| Exp. | Body weight at | | | Period of injections days | Wt. of both adrenals mg | Wt. of liver in % of body wt. |
|---|---|---|---|---|---|---|
| | castr. g | hyp.ect. g | exam. g | | | |
| 1 | 55 | 165 | 150 | 13 | 9.0 | 3.17 |
| 2 | 55 | 95 | 105 | 16 | 6.0 | — |
| 3 | 60 | 95 | 115 | 20 | 6.0 | 3.83 |
| 4 | 85 | 190 | 175 | 27 | 9.0 | 2.86 |
| 5 | 80 | 170 | 150 | 27 | 10.0 | 3.67 |
| 6 | 85 | 205 | 185 | 29 | 12.5 | 2.97 |
| 7 | 90 | 215 | 200 | 31 | 8.6 | 3.00 |

*Group IV b:* (table 4). With regard to the age of the rats at castration and to the time between castration, hypophysectomy and start of injections the present group of 7 rats corresponds to group III b. As in group III b injections of oestrone and progesterone were given. Instead of zinc insulin lente a preparation of a similar composition but without insulin activity, "lente minus insulin", was administered. In none of these experiments did the mammary gland tissues proliferate to a degree comparable with the corresponding insulin group III b. Growth processes were absent or slight, as in group II (compare figs. 8 and 2). Two rats gained and the others lost on an average 9 % in body weight after hypophysectomy. The mean weight of the adrenal glands, $8.73 \pm 0.86$ (7), was, if anything, lower than that of group II ($0.1 > P > 0.05$). The liver weight in per cent of body weight was approximately the same as in group II.

*Group V:* 13 rats castrated and hypophysectomized at an age corresponding to that in the foregoing groups were injected with insulin alone, 7 with protamine zinc insulin and 6 with zinc insulin lente. Treatment was instituted 3 weeks after hypophysectomy. The mammary glands, examined 9 to 23 days after the start of the treatment, presented roughly the same picture as before the administration of insulin, but in some glands the ducts seemed to be slightly distended (figs. 9 and 10, plate 2). During the injections the weight of the body as well as that of the liver increased markedly. The mean weight of the liver in per cent of body weight was $4.7 \pm 0.3$ (13).

In addition to the five groups described brief mention should be made of experiments in which the oestrone was applied locally to three of the mammary glands on one side. One drop, approximately 0.01 ml of oestrone in arachis oil (1,000 $\mu$g per ml) was given daily onto the nipple of each gland. The times chosen for the castration, hypophysectomy and beginning of treatment were roughly the same as in the experiments reported on above, but the dose of insulin administered was doubled every other day. Except for one rat which survived 15 days all of the 10 others receiving this treatment died within 11 days, most of them at about the 10th day. Examination of the mammary glands 9 to 15 days after the start of the treatment revealed a marked proliferation of ducts in all rats, seven of which had been injected with protamine zinc insulin and the remaining rats with zinc insulin lente (fig. 12, plate 2). Growth was found in these experiments in the oestrone treated as well as in the untreated mammary glands, and the vagina and uterus presented a state of oestrus. In 5 hypophysectomized controls not injected with insulin the local application of oestrone did not promote any growth of the mammary glands (fig. 11, plate 2). Marked growth was, however, obtained in 5 castrated controls with intact pituitary gland.

## Discussion.

The observations made in the present work with regard to the tolerance of hypophysectomized rats to long-acting insulin as well as to the effect of this agent on the weight of the body, liver and adrenal glands confirm those of SALTER and BEST (1953). The highest doses of insulin used in our experiments were, however, about 5 times those given by SALTER and BEST. The reaction of the mammary gland to ovarian hormones has not been studied before in hypophysectomized rats injected with long-acting insulin. The results obtained from group III as well as from those with local application of oestrone show that the mammary gland tissue can, by means of insulin administration, be made responsive to the action of oestrone and, possibly, progesterone in the absence of anterior pituitary hormones. From the experiments on the castrated controls with intact pituitary gland (group I) it must be concluded that the dose of progesterone used in the present work was too small to promote a proliferation of acini. The findings made on group IV show that the growth and development of the

mammary gland tissues observed in group III cannot be due to constituents of the preparations other than insulin, supplemented perhaps by glucagon. As pointed out above, the effectiveness of protamine zinc insulin seemed to be slightly higher than that of insulin lente. Further work is, however, necessary to investigate this point. The weight of the body and the liver increased in the rats of group V, injected with insulin alone, to the same extent as in those of group III, but the mammary glands did not develop as in group III. There was certainly no allometric (SILVER 1953) growth in group V, but conclusions as to a possible occurrence of isometric growth should await further experiments on rats younger than those used in the present work. In this connection it shall be emphasized that all rats used for the present experiments were immature at castration, that a slight development of the mammary glands did occur after castration and that the hypophysectomy was done before the animal was fully grown.

It seems tempting to speculate about the nature of the stimulating action exerted by insulin on the responsiveness of the mammary gland to ovarian hormones, and work is in progress to test several alternatives. The insulin action might be exerted locally on the mammary gland cells. BALMAIN and FOLLEY (1951) demonstrated a stimulating effect of insulin on mammary gland slices from lactating rats. This effect was, however, not obtained in non-lactating mammary glands. CLARINGBOLD (1954) found in mice that the effect of oestrone on the vagina can be increased by insulin and that this is most likely due to a local action of insulin on the vaginal epithelium. With regard to the high doses of insulin administered in the present experiments the possibility of a similar action on the cells of the mammary gland must be taken into account. However, in the absence of anterior pituitary hormones the vagina remains responsive to the action of ovarian hormones but the mammary gland does not.

As judged from the increase in body weight and length, and from the nitrogen retention in the hypophysectomized rat the effect of insulin (SALTER and BEST 1953, LAWRENCE, SALTER and BEST 1954) which includes an increased voluntary food intake seems to be similar to that of force feeding (SAMUELS, REINECKE and BAUMAN 1943). In force fed hypophysectomized rats the mammary glands do not respond to ovarian hormones (SAMUELS et al. 1941). In the present experiments they did. In view of the fact that insulin acts as a powerful stimulus to protein anabolism

it seems reasonable to assume that the procedure used by SALTER and BEST is more efficient in altering the pathways of the intermediary metabolism than force feeding, and that the mammary gland is a more sensitive and specific indicator of this difference than the criteria used by the two groups of workers. Thus the possibility has to be kept in mind that the action of insulin on the mammary gland might be mediated by some general metabolic change. As judged from the weight and the microscopic appearance of the adrenal glands a participation of adrenocortical steroids seems unlikely.

Whatever the mode of action of insulin on the mammary gland of the hypophysectomized rat, it seems interesting that evidence has been presented indicating 1) that the liberation of insulin into the blood is reduced after hypophysectomy in rats (RANDLE 1955), 2) that growth hormone and, possibly also, prolactin may enhance the secretion of insulin from the pancreatic islets (MILMAN, DEMOOR and LUKENS 1951, FOÀ et al. 1955, LUKENS and MCCANN 1955) and 3) that growth hormone and prolactin stimulate the responsiveness of the mammary gland to ovarian hormones in hypophysectomized rats (LYONS 1951).

## Summary.

The action of insulin on the responsiveness of the mammary gland to ovarian hormones has been studied in hypophysectomized immature female rats.

Protamine zinc insulin as well as zinc insulin lente exerted a stimulating action. The mammary glands of rats that had received insulin, oestrone and progesterone showed growth and differentiation in spite of the absence of the anterior pituitary gland. Such allometric growth was not produced by insulin given without oestrone and progesterone.

Control solutions, a protamine zinc suspension and a "lente minus insulin" preparation were ineffective.

The nature of this insulin effect and its possible relationship to the action of growth hormone on the mammary gland are discussed.

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## Comments to Plates I and II.

The figures show photographs of whole mount preparations of second thoracic mammary glands stained with gallocyanin chromalum. The magnification is the same in all figures. The mammary glands were chosen from the various groups of the present experiments. Details concerning the experimental group and the procedures performed on the animal from which the gland was obtained are given in the description of each figure.

## Plate I.

Fig. 1: (Group I) Castrated rat with intact pituitary gland. Injections of oestrone and progesterone. Age at castration 21 days. Start of treatment 52 days after castration. Examination 16 days after the first injection.

The gland shows growth and development of side buds and end buds. Very few alveoli.

Fig. 2: (Group II) Hypophysectomized castrated rat. Injections of oestrone and progesterone. Age at castration 32 days. Age at hypophysectomy (hyp.ect.) 69 days. Start of treatment 19 days after hyp.ect. Examination 16 days after the first injection.

Atrophic gland.

Fig. 3: (Group III, table 1, exp. 7) Hypophysectomized castrated rat, injected with oestrone, progesterone and protamine zinc insulin. Age at castration 21 days. Age at hyp.ect. 51 days. Start of treatment 22 days after hyp.ect.

Control gland extirpated 19 days after hyp.ect. (3 days before start of treatment).

Fig. 4: same as in fig. 3; 17 days after the first injection.

Growth and development of side buds and end buds. Very few alveoli.

Fig. 5: (Group IV a, table 3, exp. 8) Hypophysectomized castrated rat injected with oestrone, progesterone and protamine zinc suspension. Age at castration 21 days. Age at hyp.ect. 55 days. Start of treatment 19 days after hyp.ect. Examination 23 days after the first injection.

Atrophic gland with a few end buds.

Fig. 6: (Group III b, table 2, exp. 4) Hypophysectomized castrated rat injected with oestrone, progesterone and zinc insulin lente. Age at castration 23 days. Age at hyp.ect. 37 days. Start of treatment 47 days after hyp.ect.

Control gland extirpated 46 days after hyp.ect. (1 day before start of treatment).

Fig. 7: same as in fig. 6; 10 days after the first injection.

Growth and development of side buds and end buds.

## Plate I.

Fig. 1.

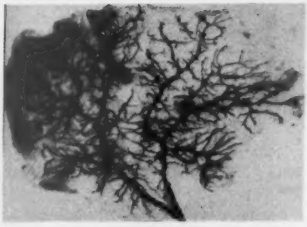


Fig. 2.

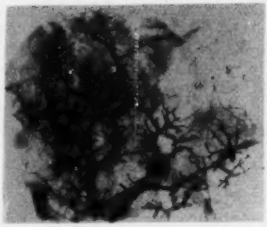


Fig. 3.

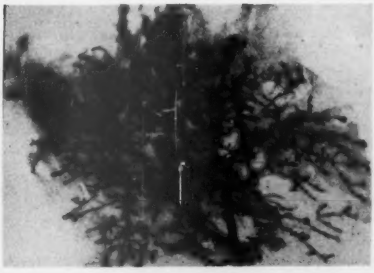


Fig. 4.

Fig. 5.

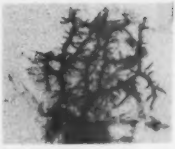
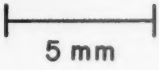


Fig. 6.

Fig. 7.




AHRÉN, JACOBSOHN: Mammary Gland Growth in Hypophysectomized Rats.

Acta Physiol. Scand. 37 : 2—3.

## Plate II.

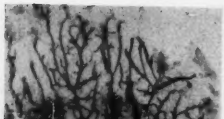


Fig. 8.

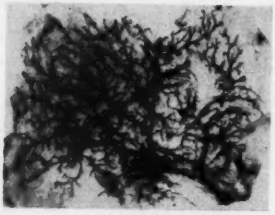


Fig. 9.

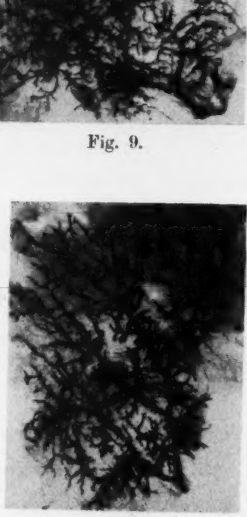


Fig. 10.

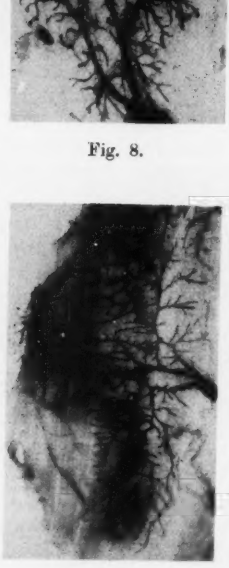


Fig. 11.

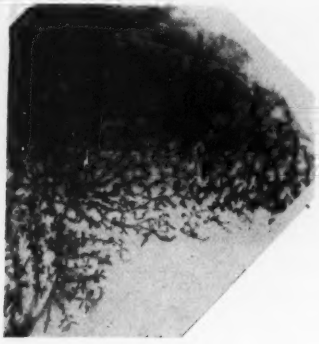
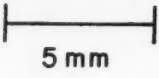


Fig. 12.



## Plate II.

Fig. 8: (Group IV b, table 4, exp. 7) Hypophysectomized castrated rat injected with oestrone, progesterone and "lente minus insulin". Age at castration 32 days. Age at hyp.ect. 55 days. Start of treatment 19 days after hyp.ect. Examination 31 days after the first injection.

Atrophic gland.

Fig. 9: (Group V) Hypophysectomized castrated rat injected with protamine zinc insulin. Age at castration 33 days. Age at hyp.ect. 71 days. Start of treatment 20 days after hyp.ect.

Control gland extirpated 18 days after hyp.ect. (2 days before start of treatment).

Fig. 10: same as in fig. 9; 14 days after the first injection.

Slight thickening of ducts.

Fig. 11: Hypophysectomized castrated rat. Local application of oestrone on the mammary gland and intramuscular injections of progesterone. Age at castration 68 days. Age at hyp.ect. 75 days. Start of treatment 6 days after hyp.ect. Examination 16 days after the first injection.

Atrophic gland.

Fig. 12: Hypophysectomized castrated rat. Local application of oestrone on the mammary gland, intramuscular injections of progesterone and injections of protamine zinc insulin subcutaneously. Age at castration 68 days. Age at hyp.ect. 74 days. Start of treatment 7 days after hyp.ect. Examination 10 days after the first injection.

Growth and development of side buds, end buds and some alveoli. The photograph shows approximately half of this gland.

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# Shock Wave Transmission to the Central Nervous System.

By

CARL-JOHAN CLEMEDSON.

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Involvement of the central nervous system in severe blast injury has been described by a number of authors (see *e. g.* MAIRET and DURANTE 1919, KING and CURTIS 1942, DENNY-BROWN 1945 and CLEMEDSON 1949). Originally the greatest stress was laid upon the changes found in the nervous system and the blast injury problem was supposed to be essentially a neurological one. Nowadays the preponderance of the lung lesions, which was first demonstrated by RUSCA (1914) and HOOKER (1924), is generally recognized but it is still agreed that exposure to a severe blast may cause a number of pathological changes in the nervous system.

In brief, the changes reported are hemorrhages in the meninges, brain substance and nerve roots (RUSCA 1914, MOTT 1916, MAIRET and DURANTE 1919, STEWART, RUSSEL and CONE 1941 and ASCROFT 1943) and early degenerative processes in the ganglia, cortex and the subcortical parts of the white matter (PINES and SHEFTELJ 1937, ZURABASHVILI 1945). CLEMEDSON, in an earlier investigation (1949), observed meningeal congestion, a sometimes intense brain edema and blood clots in the cisterns in severely blast injured rabbits, but in no case macroscopically noticeable hemorrhages in the substance of the brain or the spinal marrow were found.

The biophysical mechanism of the changes in the central nervous system and the transmission of shock wave energy to that part of the body has been subject to only very incomplete treatment in the literature. It has been assumed, although never proved experimentally, that the shock wave is transmitted to the critical brain regions through the blood vessels (MEIGRE 1916, MOTT 1916, STEWART et coll. 1941, YOUNG 1945) and by the cerebrospinal fluid (MEIGRE 1916, HAMLIN 1943, YOUNG 1945, SHPILBERG 1945). YOUNG who has by means of constricting broad canvas bands around the abdomen and the thorax of dogs tried to simulate and visualize "in slow motion" the effects of increased pressure on the body, assumes that during the blast exposure the pressure is increased on all the surfaces and fluids of the body with the exception of the craniovertebral space. Within the cranium the pressure should, he presumes, be lower than in any other part in the body and the blood should therefore be pressed into this area and exert a hydrostatic pressure on the brain and spinal marrow.

In an earlier investigation (CLEMEDSON and PETTERSSON 1956) it was found that high explosive shock waves are propagated through the brain. The way of transmission to the brain was not studied, but the fact that shock waves are easily transmitted through bone tissue (CLEMEDSON and PETTERSSON 1955) makes it highly probable that the direct, transosteal transmission is the most important way for the shock wave energy to reach the brain. The present investigation was undertaken in order to study the mechanism of shock wave transmission to the brain and spinal marrow.

## Methods.

Rabbits weighing 2.5—3 kg were used as experimental animals. Due to the size of the pressure transducers used, the experiments had to be performed in dead animals. They were sacrificed by a rapid injection of 3 cc of the barbiturate anesthetic Narkotal. To prevent clotting 1.5 cc of Heparin were given 5 min before the injection of Narkotal.

The animals were exposed to high explosive shock waves in the detonation chamber described earlier (CLEMEDSON 1949, CLEMEDSON and CRIBORN 1955 a). The charge used was an electric blasting cap and 1 g of Pentyl (PETN), a high explosive, the chief constituent of which is pentaerytrittetranitrate. Peak pressure and impulse determinations in the incident shock wave were made in each blasting according to methods earlier described (CLEMEDSON and CRIBORN 1955 a).

The shock wave transmitted to the brain was recorded by means of a barium titanate pressure transducer. The transducer model BC-10 made by Atlantic Research Corp., Alexandria, Va. U. S. A. was used throughout this experimental series. The shape of the transducer is cylindrical and it has a length of about 30 mm and a diameter of 9 mm. The sensitivity of the two transducers employed are 0.099 and 0.094 volts/psi respectively. Their frequency ranges from 3 c/sec to 75 kc/sec and they show a linear response for pressures up to about 10,000 psi, *i. e.* pressures very much higher than those obtained in the experiments to be described here. The properties of the transducer such as its time constant and its response to a shock pulse of known shape have been tested earlier and have been found to be satisfactory for a correct reproduction of the shock waves produced in the detonation chamber (CLEMEDSON and PETTERSSON 1956).

As recording apparatus a Tektronix cathode ray oscillograph type 535 was employed. The transducer voltage was amplified by a wide-band, high-gain calibrated plug-in type preamplifier type 53 B in the oscillograph. The input resistance of the oscillograph is 1 meg. and the capacity of the transducer 7,000 pF. A DuMont polaroid oscillograph recording camera was attached to the oscillograph. The synchronization of the release of the sweep of the oscillograph with the detonation was accomplished by means of an aluminium foil contact device placed between the charge and the animal and about 25 cm from the latter.

The transducer was inserted into the brain in the following way. The left eye was removed and the optic foramen enlarged. The transducer was introduced and thoroughly fixed by sutures. The orbit was filled with cotton-wool around the transducer cable and finally the orbit was closed by sutures through the eyelids.

In order to detect any possible different ways of shock wave transmission to the brain the animals were exposed in the following three different ways *viz.* with the whole body exposed to the shock wave, with the whole body except the head protected from the shock wave and with only the head protected. Protection was accomplished by means of inclusion of the parts of the body to be protected in a metal cylinder with thick walls. To ensure the best possible protection, the part to be protected was thoroughly wrapped with cotton-wool and put in the metal cylinder. The cylinder then was surrounded by a layer of cotton-wool at least 10 cm thick. Outside this layer sheets of foam rubber were fixed. Special attention was paid to the packing around the neck of the animal where it protruded from the metal cylinder. To test the effectiveness of the protection, transducer recordings were also taken with both the head and the rest of the body protected at the same time.

In some experiments the shock wave transmission through the spinal column was investigated both with the head exposed and the rest of the body protected (propagation from head down through the spine) and with the body exposed with the exception of the head (propagation from spine to head).

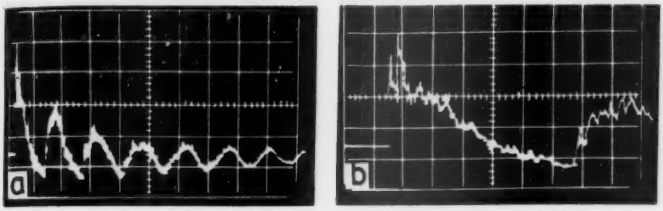


Fig. 1. Recordings of pressure pattern in incident air shock wave. Time (between vertical lines): a 5 msec and b 1 msec. Pressure sensitivity (between horizontal lines): 0.35 kp/cm² in Figs. 1—3.

Due to the relatively low blast pressures obtained with a charge of only 1 g of PETN the destructive effects of the shock wave were negligible and each animal could therefore be used for several blastings, usually for two exposures with each of the four different types of protection (no protection, head protected, body except head protected and total body protection).

## Results.

The results to be reported here are based on about 140 blast experiments in the detonation chamber.

The general characteristics of the air shock wave in the detonation chamber just before the impact against the animal's head is seen in Fig. 1. Fig. 1 a shows the sequence of pressure peaks with diminishing amplitude generated by the charge in the closed detonation chamber. The details of the first and the beginning of the second peak can be studied in Fig. 1 b. The complex multi-peak shock wave pattern obtained in the detonation chamber has been described elsewhere (CLEMEDSON and CRIBORN 1955 a and b, CLEMEDSON and PETTERSSON 1956) and will therefore not be dealt with here.

The characteristic shock wave patterns recorded in the brain with different parts of the body protected against the incident shock wave are found in Fig. 2. If the whole body is exposed, *i. e.* without any protection at all, a curve such as Fig. 1 a is obtained. Curve b recorded with shorter sweep time shows the first pressure peak in detail. A comparison with Fig. 1 shows that the wave transmitted to the brain does not differ very much from the incident shock wave in air. The curves 2 c and 2 d are obtained with the head of the animal exposed to the shock wave but the rest of its body thoroughly protected. The amplitude of the first peak is the same and except a somewhat

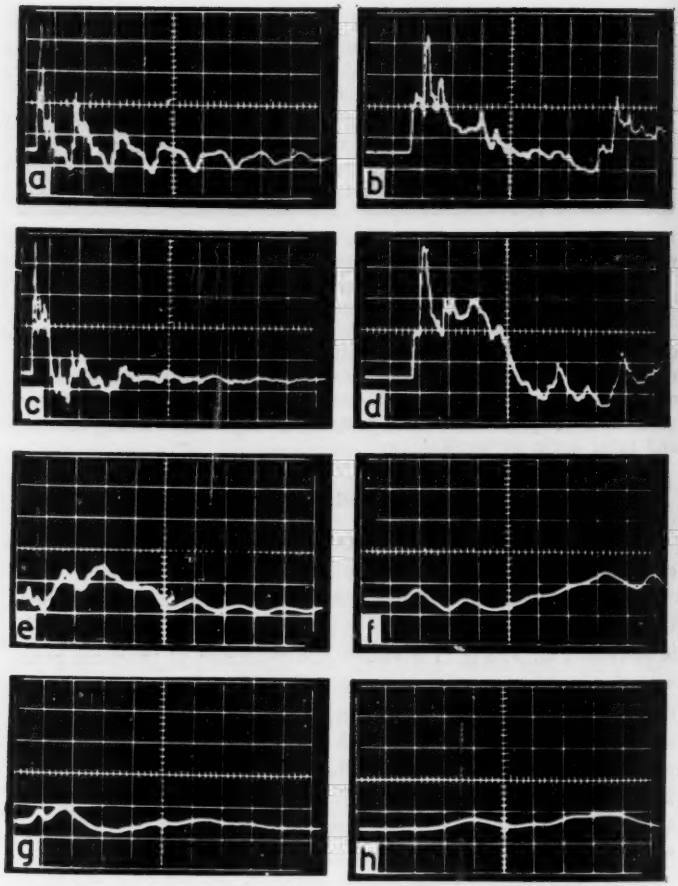


Fig. 2. Pressure wave recordings in the brain. a and b in an unprotected animal; c and d with the trunk protected; e and f with the head protected and g and h with the whole animal protected. Time: a, c, e and g 5 msec; b, d, f and h 1 msec.

slower decrease of the pressure this peak is very much the same as that obtained in the unprotected animal. The following peaks in curve c, however, have smaller amplitude than in curve a and it is obvious that the pressure peaks pass away more rapidly if the trunk and the neck of the animal are protected.

If the head of the animal is protected but the rest of the body exposed to the shock wave curves like those in Fig. 2 e and f

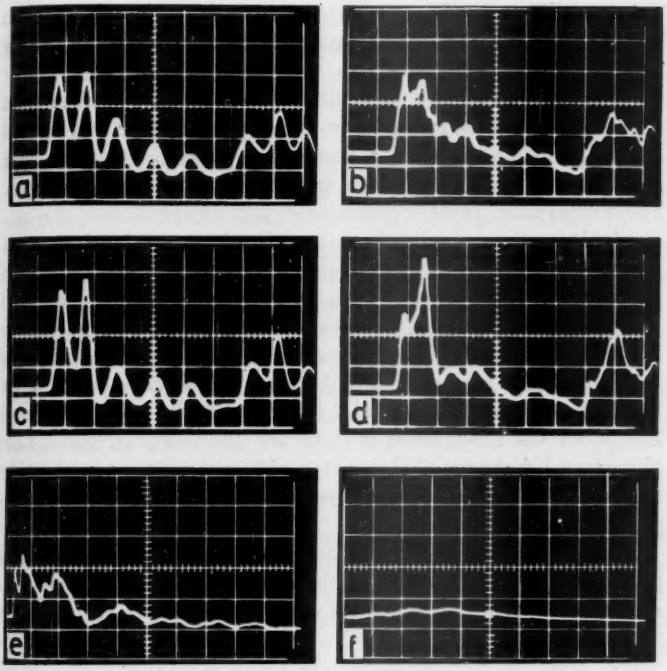


Fig. 3. Pressure wave recordings in the spine. Curves a, c and e are recorded in the spine, curves b, d and f at the same level in the abdominal cavity. Time: 1 msec in curves a—f.

are obtained. These curves are of quite another type. It is evident that there is some pressure transmitted to the brain even if the skull is thoroughly protected from direct impact by the shock wave. The sharp peaks are, however, completely lacking and only smaller pressure oscillations are propagated to the brain. The actual pressure transmitted in this way might be even less, as it is seen from curves g and h, recorded with total protection of the whole animal, that despite a thorough complete protection some pressure is nevertheless propagated to the transducer.

To test further the possible significance of the vascular communication between the thorax and the brain for a transmission of shock pressure the blood vessels of the neck were compressed by means of a rubber band which was firmly tightened around the neck. The shock waves recorded in the brains of such animals

did not differ significantly, however, from those obtained in animals with intact vascular communication between the thorax and the brain.

In order to study the possibility of a shock wave transmission from the skull to the spinal marrow the spinal column was opened in the lumbal region and a transducer placed in contact with the spinal cord. The transducer was fixed and the wounds closed. For comparison another transducer was inserted into the abdominal cavity and placed at exactly the same level as that in the spinal column. Fig. 3 shows the results from such an experiment. The curves a, c and e are from the spinal column and the curves b, d and f are the corresponding pressure curves in the abdominal cavity. The curves a and b were recorded in an unprotected animal, c and d in the same animal with only its head protected and e and f finally with the body protected except for the head. Even if there are differences in the details the general features of the curves obtained with no protection and of those with the head protected are, which is to be expected, essentially the same.

If the neck and trunk are protected, hardly any pressure is recorded by the transducer in the abdominal cavity. The transducer in contact with the spine on the other hand, nevertheless indicates the transmission of a pressure wave. This pressure wave, however, has an amplitude which is smaller than that of the brain curve and is considerably changed. It must have been propagated through the spinal column from the head. Contrary to what could be expected the pressure curves obtained in the spine display less pressure oscillations of high frequencies than those recorded in the abdomen. This is probably due to the difficulty to achieve a sufficiently good contact between the comparatively big transducer and the spinal column and marrow.

The experiments described above thus have given evidence for a certain degree of pressure transmission from the head down through the spinal column. In order to test, if also the reverse way of propagation takes place, some animals were exposed with the transducer in the brain and the head protected after complete transsection of the cervical spinal column and separation of its two cut ends by means of cotton-wool. The fact that the pressure curves obtained in the brain with the spinal column intact (Fig. 2 e and f) and with the spine separated from the head are almost identical, indicates that there is no significant pressure wave

transmission from the body through the spine to the head at least at the relatively low shock pressures used in this investigation.

As the animal cage is fixed on the impulse pendulum of the detonation chamber (see CLEMEDSON 1949 and CLEMEDSON and CRIBORN 1955 a) the animal is brought with the pendulum body when it is flung out by the detonation. In order to detect any effects on the pressure curves in the brain due to a possible rapid movement of blood masses to or from the brain caused by the acceleration of the animal, exposures were made both with the head and the tail end of the animal turned to the charge. The pressure curves were somewhat different in the two types of experiments due to the fact that the general shock wave pattern differs at various levels in the detonation chamber. It was, however, evident from these experiments that the position of the animal had no significant influence on the results of the pressure recordings in the brain.

## Discussion.

It has been known since long that the shock wave from a detonating high explosive can be transmitted to and cause severe injuries in the central nervous system. The mechanism of transmission of the shock wave energy to the brain has not been dealt with experimentally before but it has been assumed by some authors that the effects on the central nervous system were caused by an increased intrathoracic pressure which should be propagated through the blood vessels. YOUNG (1945) states that at the moment of detonation due to the bony encasement of the brain the pressure within the skull should be the lowest in the body and therefore the blood from other areas is "literally squeezed into this low pressure area in the cranium which becomes quickly filled with fluid and then experiences the maximum force of the external pressure according to Pascal's hydrostatic law". This law states that a pressure exerted anywhere on a liquid mass is transmitted undiminished in all directions and acts with the same force on all equal surfaces and in a direction at right angles to the surfaces. According to YOUNG the tremendous hydrostatic pressure action could well explain the pathologic changes in the brain and also the instantaneous death sometimes seen in severe blast.

The shock wave impact, indeed, causes a sharp but very short-lasting rise of intravascular pressure (CLEMEDSON 1949). It seemed,

however, rather unlikely that this short increase in blood pressure could be the chief cause of the characteristic large pressure peaks observed by CLEMEDSON and PETTERSSON (1956) in rabbits exposed to shock waves in the detonation chamber. Furthermore, the brain is normally protected from damage through violent pulse wave pressures thanks to the anatomical arrangement of the brain vessels, the special structure of the brain arteries and other mechanisms which have been reviewed recently by ASK-UPMARK (1955).

The other possible mechanism, the direct transosteal propagation of the shock wave, has not been discussed in the literature but it must a priori be assumed to be the more plausible one. Thus, it has been shown that the skull bones easily transmit shock waves (HARVEY and MCMILLEN 1947) and that shock waves will be propagated through fairly thick bone tissue without any appreciable weakening.

The present investigation has clearly evidenced that the shock wave energy is essentially transmitted to the central nervous system directly through the surrounding tissues. There is, indeed, a propagation of a pressure wave to the brain even if the skull is completely protected but this wave is of considerably smaller amplitude and is much more damped as compared with the directly transmitted one. This wave is transmitted through the various tissues of the neck, but it has not been possible to show in this investigation that special structures for example the blood vessels or the spinal column are more important in that respect than other body tissues. It should, however, be kept in mind that, owing to the dimensions of the pressure transducer, the experiments had to be performed in dead animals. In living animals with normal hemodynamic conditions the conditions for a pressure wave transmission through the blood vessels may be somewhat different but it certainly could not change the result that the majority of the incident shock wave pressure is transmitted the direct transosteal way to the brain.

The passage of the shock wave through the skull of a rabbit will cause only minor changes of the amplitudes and the general shape of the waves. Our factual knowledge of the propagation of shock or pressure waves in various biological structures is, however, far too incomplete to allow a satisfactory explanation of the differences observed between the incident air shock wave and the brain transmitted one and between the pressure wave

pattern in the brains of animals without protection and in those with their trunks protected.

This investigation has also shown that even if the shock wave hits the head only, part of the pressure is transmitted to and propagated through the spinal column. Due to the size of the pressure transducer, it has unfortunately not been possible to establish the significance of the various structures, the spinal column, the cerebrospinal fluid or the spinal marrow for that transmission. It is somewhat surprising, however, that there is less ability for a transmission in the opposite direction. The finding is nevertheless of great practical interest as it speaks against the possibilities of a more severe cerebral involvement in a man exposed to an underwater blast if only his head is well over the water surface at the moment of detonation.

## Summary.

The transmission of air shock waves to the central nervous system was studied in rabbits by means of a barium titanate pressure transducer inserted into the brain or the spinal column.

By exposing only a part of the animal *e. g.* the head only or the trunk to the shock wave and with the other part thoroughly protected, it was possible to show that the main part of the shock wave pressure is transmitted to the brain directly through the skull. The indirect transmission of pressure from other parts of the body to the brain is of minor importance and the rôle of the blood vessels or the spine for that transmission is insignificant.

Pressure is also propagated from the skull down through the spine but the transmission in the opposite direction, when the abdomen only is exposed is insignificant.

*Acknowledgement.* The skilful technical assistance of Mr. Arne Jönsson is gratefully acknowledged.

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# On the Permeability of Guinea Pig Placenta for Triiodothyronine.

By

HARRY LYBECK.

Received 28 April 1956.

Recent studies with thyroxine labeled with radioactive iodine ($I^{131}$) indicate that it does not permeate the guinea-pig placenta in the first hours after its administration either in the direction from the mother animal to the foetuses or vice versa. (LYBECK and HIRVONEN 1955). In 1952, a new iodinated amino acid, 3,5,3''-triiodothyronine, was found by GROSS and PITT-RIVERS (1952) in the human plasma and in hydrolysates of the thyroid. This new substance has been shown to possess even more biological activity than thyroxine (GROSS and PITT-RIVERS 1952, 1953, GEMMILL 1953, HIRVONEN and LYBECK 1955). The biological activity of this compound determined on the basis of rate acceleration in the isolated rat auricle gives a ratio of 10 : 1 in favour of triiodothyronine (HIRVONEN and LYBECK 1955). It has also been postulated that triiodothyronine may arise from thyroxine by deiodination of the thyroxine molecule in the tissues, and that this new compound is probably the true hormone of the thyroid. It was therefore considered of interest to investigate whether this "new hormone", structurally so like thyroxine, would behave like it in relation to the placenta.

## Methods.

Nine guinea-pigs in late pregnancy with 34 foetuses weighing from 30 to 103 g served as experimental animals. Radioactive triiodothyronine labeled with $I^{131}$ by exchange was freshly prepared and identified chromatographically (LYBECK 1954). The radioactive substances were injected subcutaneously in the nape of the neck as a faintly alkaline so-

lution. The activity of the dose ranged from 10—15 microcuries. After a time interval of about 2 to 4 hrs from the administration of the radioactive material, the animals were anaesthetized with an intraperitonial Nembutal injection and the foetuses were removed from the uterus.

The following liquid samples were taken, prepared and counted: amniotic fluid, foetal and maternal blood by heart puncture and maternal bile.

The counting equipment used consisted of a well type Geiger-Müller counter with electronic scale of eight.

## Results.

The observations on the radioactivity of the samples from all the experiments are shown in Table I. Two to four hours after the subcutaneous administration of the radioactive triiodothyronine, a significant amount of radioactivity was detectable in all the samples from the mother animals. Radioactivity was also demonstrable in the foetal samples but it was very feeble. Only in one experiment (No. 5) did it exceed twice the mean value of the background; in all the others it was much less than that.

There is a marked difference between the radioactivity values of the maternal blood samples. In eight experiments out of nine the values of the maternal blood samples exceeded those of the foetal blood samples by a factor of at least five and in most cases considerably higher. In but one experiment (No. 2) was the radioactivity of the foetal blood samples only about three times that of the maternal blood samples. The radioactivity of the amniotic fluid was approximately equal to the radioactivity of the foetal blood.

The highest values were obtained for the maternal bile. They varied from two to six times the values of the corresponding maternal blood samples.

## Discussion.

That inorganic iodine easily passes across the placenta in both directions is well established. In similar test conditions to above, radioactive iodine is demonstrable in great amounts in the foetal blood (Lybeck and Hirvonen 1955). The results of the present investigation show that this is not the case with the radioactive triiodothyronine.

During the time intervals used in the present experiments radioactive triiodothyronine circulates in the maternal blood stream.

**Table I.**

*Experiments with radioactive triiodothyronine.*

| Mother Animal | | | | | Foetuses | | | |
|---|---|---|---|---|---|---|---|---|
| No. | Time Interval (hr.) | Wt (g) | Radioactivity cpm | | No. | Wt (g) | Radioactivity cpm | |
| | | | 0.25 cc Blood | 0.25 cc Bile | | | 0.25 cc Blood | 0.25 cc Amniotic Fluid. |
| 1 | 2 | 1,105 | 198 | 805 | I<br>II<br>III<br>IV | 67<br>58<br>71<br>62 | 40<br>26<br>31<br>34 | 19<br>18<br>11<br>35 |
| 2 | 2½ | 1,070 | 103 | 220 | I<br>II<br>III | 59<br>70<br>62 | 34<br>35<br>29 | 31<br>36<br>29 |
| 3 | 3 | 1,110 | 172 | 456 | I<br>II<br>III<br>IV<br>V<br>VI | 63<br>56<br>60<br>49<br>62<br>30 | 11<br>8<br><br>16<br>13 | 5<br>19<br>13<br>31<br>15 |
| 4 | 3 | 985 | 129 | 407 | I<br>II | 64<br>66 | 18<br>26 | 14<br>31 |
| 5 | 4 | 1,180 | 413 | 2,089 | I<br>II<br>III<br>IV<br>V | 45<br>49<br>51<br>54<br>65 | 62<br>52<br>53<br>74<br>66 | 50<br>63<br>53<br>56<br>61 |
| 6 | 4 | 1,040 | 217 | 863 | I<br>II | 73<br>65 | 16<br>14 | 18<br>10 |
| 7 | 4 | 1,185 | 192 | 691 | I<br>II<br>III<br>IV | 63<br>63<br>67<br>58 | 13<br>21<br>6<br>15 | 9<br><br>2<br>6 |
| 8 | 4 | 1,130 | 91 | 262 | I<br>II<br>III<br>IV | 39<br>39<br>36<br>31 | 16<br>14<br>15<br>11 | 23<br>18<br>8<br>10 |
| 9 | 4 | 1,075 | 89 | 274 | I<br>II<br>III<br>IV | 30<br>30<br>32<br>30 | 12<br>14<br>11<br>13 | 11<br>8<br>10<br>11 |

A proof of this is the high radioactivity in the maternal bile samples. Nevertheless only a slight radioactivity is detectable in the samples from the foetuses, even four hours after subcutaneous administration of the radioactive substance. This indicates that only a minute amount of the radioactive material has penetrated the placenta and entered the foetal circulation.

Evidently the placenta forms an obstacle to triiodothyronine as it does to thyroxine. The latter is also held back by the placental barrier, at least during the first hours after subcutaneous (HIRVONEN and LYBECK 1955) or intravenous (LYBECK, unpublished) administration.

Present evidence indicates that triiodothyronine, like thyroxine, is loosely bound to the plasma proteins and probably to a single plasma constituent with electrophoretic mobility close to that of the alpha-globuline (DEISS, ALBRIGHT and LARSON 1953, BARAC, BRULL, LYBECK and NIZET 1955). It is imaginable that the placenta is virtually impermeable to this iodothyronine-protein complex. It is also conceivable that the exceptional molecular configuration of thyroxine and its close derivative triiodothyronine to some extent accounts for why the placenta does not allow these substances to pass readily into the foetal circulation. All the other hormones are built up of elements with fairly low atomic weights. The atomic weight of iodine is very high, 127, in comparison with the other elements of biological significance, which have atomic weights below 56.

## Summary.

The permeability of the guinea-pig placenta to $I^{131}$ labeled triiodothyronine was investigated. Two to four hours after subcutaneous administration of the substance, samples were taken and counted. A significant difference in radioactivity was demonstrable between the maternal and foetal samples. The results indicate that the placenta is virtually impermeable to exogenous triiodothyronine in the first hours after its subcutaneous administration.

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# The Fate of the Sulfate Group of Chondroitin Sulfate after Administration to Rats.

By

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Metachromatic material is known to occur in normal human urine (ASTRUP 1947). The metachromatic fraction was further investigated by KERBY (1954), who showed that the mean 24-hour excretion of acid mucopolysaccharides (expressed as glucuronic acid after acid hydrolysis) in normal human urine was 3.0 mg for the female and 4.9 for the male. She also demonstrated that the major component of a further purified fraction of acid mucopolysaccharides in normal urine behaves like chondroitin sulfate on paper chromatograms. The material was present in urine obtained from the renal pelvis and thus was not derived from structures lower in the urinary tract. These investigations suggest that chondroitin sulfate, like heparin, is able to pass the kidneys and that a small amount of a chondroitin sulfate-like substance is constantly secreted.

The fate of chondroitin sulfate and hyaluronic acid injected intravenously into rats and rabbits was studied by TINACCI and BENASSI (1954). Tissue metachromasia (rats), and urinary hexosamine levels (rabbits) were followed. In one experiment, 117 mg hexosamine was injected in the form of chondroitine sulfate, but only 31.5 per cent was recovered in the urine during the first 24 hours.

RICKETTS, WALTON and SADDINGTON (1954) investigated the metabolism of dextran ($S^{35}$) sulfate administered intravenously

to rabbits. The renal excretion was followed by measurements of the radioactivity and the metachromasia of the urine. About 30 per cent of the $S^{35}$ was eliminated in 2 days, rising to 50 per cent in 4 days. As judged by paper chromatography, the initially excreted dextran sulfate was rather low molecular. The experiments did not show whether any of the $S^{35}$ appeared in the form of inorganic sulfate.

Although much is known about the uptake of sulfate by mucopolysaccharides (reviewed by BOSTRÖM 1954), little is known about the mode of elimination of these compounds. No sulfatase attacking mucopolysaccharides has as yet been demonstrated in mammalian tissues in vitro (DOHLMAN and FRIEDENWALD 1955). The present study is concerned with the metabolism of the sulfate groups of chondroitine sulfate. Cartilage chondroitin ($S^{35}$) sulfate was prepared and administered subcutaneously and per os to rats, and the excretion in the urine, faeces, and bile was followed. Any inorganic sulfate split off from the mucopolysaccharide was separated from the chondroitin sulfate by paper electrophoresis and measured. Different kinds of tissues were also tested for sulfatase activity *in vitro*.

## Material and Methods.

### *Preparation.*

Chondroitin sulfate in cartilage was tagged *in vitro* with $S^{35}$ according to BOSTRÖM and MÅNSSON (1952). Thus slices of calf costal cartilage were incubated in Krebs' solution containing radioactive inorganic sulfate, and the chondroitin sulfate labelled in this way was subsequently isolated (BOSTRÖM and MÅNSSON 1953). Afterwards the preparation was passed repeatedly through anion exchange columns (Dowex 2, Cl-form) to remove the last traces of inorganic sulfate. When the chondroitin sulfate was tested with the paper electrophoresis method (see below), no inorganic sulfate was found.

The analytical data of the chondroitin ($S^{35}$) sulfate preparation were the following, calculated for water free substance: ash 22.0 per cent, nitrogen 3.09 per cent, sulfur 5.1 per cent, galactosamine (GARDELL 1953) 35.0 per cent, glucosamine (GARDELL 1953) 0.7 per cent and uronic acid (TRACEY 1948) 38.2 per cent. The material contained 19.15 per cent moisture.

The specific activity of the chondroitin sulfate was 47,200 counts/min/mg.

In one experiment (reported in table VII), another preparation of chondroitin sulfate was used. It had a specific activity of 86,600 counts/min/mg.

**Table I.**

*Recovery of $S^{35}$ in urine and faeces after oral administration of labelled chondroitin sulfate to rats.*

| Amount of chondroitin sulfate administered | Number of rats | Hours | Urine | | | Faeces CPM |
|---|---|---|---|---|---|---|
| | | | Total radio-activity CPM | Per cent dialyzable radio-activity | Per cent $S^{35}$ as in-organic sulfate | |
| 25 mg 587,000 CPM | 1 | 6 | 2,000 | — | — | — |
| | | 18 | 124,000 | 95.6 | 93.4 | 2,300 |
| | | 42 | 128,200 | | | 66,300 |
| | | 66 | 22,000 | | | 24,700 |
| | | 90 | 11,400 | — | — | 19,200 |
| | | 114 | 10,200 | — | — | 6,200 |
| | | | 297,800 (52.2 %) | | | 118,700 (20.8 %) |
| 65 mg 1,050,000 CPM | 1 | 6 | 60,100 | — | 98.0 | — |
| | | 18 | 475,000 | — | 98.3 | 4,500 |
| | | 42 | 253,400 | — | 95.7 | 6,900 |
| | | 66 | 21,000 | — | — | 12,500 |
| | | 90 | 7,200 | — | — | 1,000 |
| | | | 816,700 (77.8 %) | | | 24,900 (2.4 %) |

*Subcutaneous administration.*

Female rats weighing about 200 g were used. Chondroitin sulfate in varying amounts (see tables) dissolved in a few millilitres of distilled water was injected subcutaneously, and the animals were placed in restraining cages with free access to food and 0.9 per cent NaCl solution. The urine, the faeces and, in some instances, the bile, were collected and the radioactivity measured (see below).

As the faeces were readily contaminated with urine, a supra-pubic bladder fistula (polyethylene tubing) was usually established previous to the experiment, and the urethra tied off. In this way urine was collected in a flask under toluene and the faeces were collected strictly separate. Only the rats referred to in tables I and V had not bladder fistulas.

Bile fistulas were established according to Bergström, Sjövall and Voltz (1953).

*Intestinal absorption.*

Female rats with bladder fistulas were put into restraining cages as above and starved. An aqueous solution of labelled chondroitin sulfate (10—65 mg in 1—2 ml) was administered by gastric intubation

under brief ether anaesthesia. In control experiments carrier-free labelled sodium sulfate together with 30 mg of unlabelled sodium sulfate ($Na_2SO_4 \cdot 10\ H_2O$) was given in the same way. Sulfate in this concentration did not cause diarrhea.

The radioactivity of urine and faeces was measured, as reported below. In some experiments, the animals were killed at regular intervals, and the gastrointestinal tract was removed. The contents of the stomach, the small intestine and the large intestine were then separately measured for radioactivity.

Some rats were treated with antibiotics to depress the number of intestinal bacteria before administration of chondroitin sulfate. 100 mg of Terramycin and 250 mg of phthalylsulfonazolum ("Sulftalyl", Pharmacia, Sweden) together with vitamins ($B_1$, $B_2$, nicotinic acid and vitamin K) were mixed and given by stomach tube during light ether anaesthesia. This was done twice daily for four days prior to the experiment and also during the experiment. This procedure is known to diminish the amount of bacteria in the large intestine to a very small fraction of the original number (NORMAN 1955).

*In vitro incubations.*

Costal cartilage, cornea, subcutaneous tissue, liver, kidney and spleen from ox, rabbit or rat were homogenized (Bühler or Potter-Elvehjem's homogenizers) in the cold together with Krebs' phosphate buffers between pH 4.0 and 8.0. Labelled chondroitin sulfate was added and the mixtures were incubated at 38° C for various lengths of time up to 16 hours. Afterwards unlabelled inorganic sulfate carrier was added, the solutions were filtered, lyophilized and analyzed for split-off inorganic radioactive sulfate with the electrophoresis method described below.

*Paper electrophoresis and dialysis.*

The ratio of inorganic labelled sulfate to labelled chondroitin sulfate in urine and homogenates was determined after separation by paper electrophoresis. The materials were lyophilized and samples of suitable concentrations were added to paper strips (SCHLEICHER & SCHÜLL No. 2043 B) and run for 9 hours in a 0.15 molar Michaelis' buffer pH 3.1 with a current of 0.5 mA/cm filter paper. When labelled chondroitin sulfate and labelled inorganic sulfate were added to normal urine, a complete separation occurred (Fig. 1). Only one radioactive spot was found when labelled inorganic sulfate was mixed with unlabelled chondroitin sulfate in urine. Although the radioactive chondroitin sulfate peak was often less sharp in urine (sharp in buffer alone), no trailing of the inorganic sulfate ever occurred. When the samples under investigation were tested with the electrophoresis method described, peaks exactly corresponding to chondroitin sulfate and inorganic sulfate were found.

In order to determine whether the excreted chondroitin sulfate occurs in a more depolymerized form, some urine samples were dialyzed

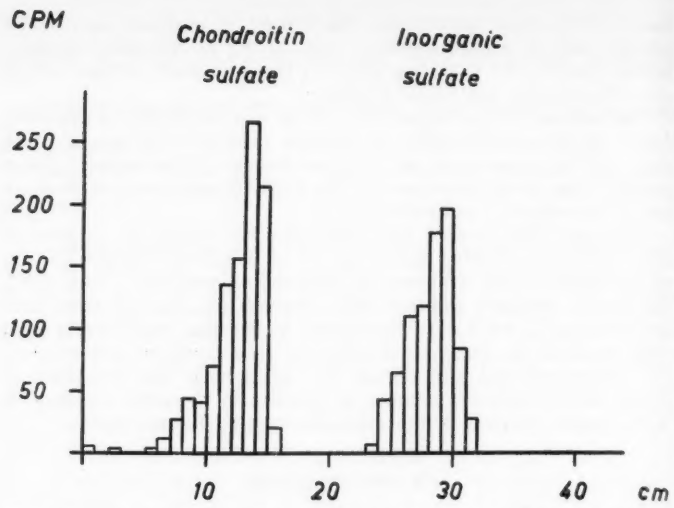


Fig. 1. Paper electrophoresis of labelled chondroitin sulfate and labelled inorganic sulfate added to normal rat urine.

through dialysis tubing and the results were correlated with the amount of free radioactive sulfate.

*Measurements of radioactivity.*

The determination of $S^{35}$ as sulfate or chondroitin sulfate was performed on samples of nearly "infinite thinness" with a thin end-window Geiger-Müller tube. Samples of filtered and diluted urine, faeces or bile were evaporated on aluminum planchets to leave less than 0.5 mg/cm$^2$.

As chondroitin sulfate tends to form complexes with the faecal proteins, reactions which may cause loss of radioactivity at the filtering, the faecal samples were often hydrolyzed with 4 N HCl and afterwards neutralized. The free sulfate could then be measured more accurately for radioactivity.

After the paper electrophoresis runs, the paper strips were dried and taped to a ruler that was automatically moved past a GM-tube. A slit in a metal plate between the paper and the mica window allowed the counting of 1 cm of the paper each time.

## Results and Discussion.

The excretion of $S^{35}$ in the urine and faeces after oral administration of labelled chondroitin sulfate is recorded in table I. Evi-

**Table II.**

*Radioactivity recovered in the urine and in various parts of the gastro-intestinal tract of rats after oral administration of labelled chondroitin sulfate (115,000 CPM).*

| Number of rats | Hours | Urine | Stomach | Small intestine | Large intestine + faeces |
|---|---|---|---|---|---|
| 1 | 3 | 20 | 2,700 | 15,000 | 91,300 |
| 2 | 3 | 350 | — | — | — |
| | 6 | 19,100 | 200 | 3,600 | 59,100 |
| 1 | 3 | 170 | — | — | — |
| | 6 | 14,600 | — | — | — |
| | 9 | 12,200 | 150 | 4,200 | 51,600 |
| 2 | 3 | 140 | — | — | — |
| | 6 | 6,200 | — | — | — |
| | 9 | 7,900 | — | — | — |
| | 12 | 11,800 | 140 | 7,400 | 55,700 |
| 1 | 3 | 130 | — | — | — |
| | 6 | 6,000 | — | — | — |
| | 9 | 16,400 | — | — | — |
| | 12 | 16,300 | — | — | — |
| | 24 | 38,500 | 130 | 1,100 | 4,800 |

dently, radioactive material was absorbed from the gastro-intestinal tract as it appeared in the urine. However, dialysis experiments and determinations of inorganic sulfate showed that most of the $S^{35}$ was excreted as inorganic sulfate rather than as chondroitin sulfate. This fact makes it improbable that intact chondroitin sulfate can be absorbed from the alimentary canal to any great extent.

The urinary excretion seemed to be characteristically retarded when compared with that following subcutaneous administration (see below). Within 6 hours only a small fraction of the total radioactivity appeared in the urine. Not until during the following 36 hours did the urinary excretion reach its peak, after which it rapidly declined. This mode of excretion suggested either that the passage through the alimentary canal was abnormally slow or that the radioactive material was not absorbed until it had reached the large intestine.

In an attempt to locate the site of absorption of the labelled material, several rats with bladder fistulas were given 5 mg (115,000

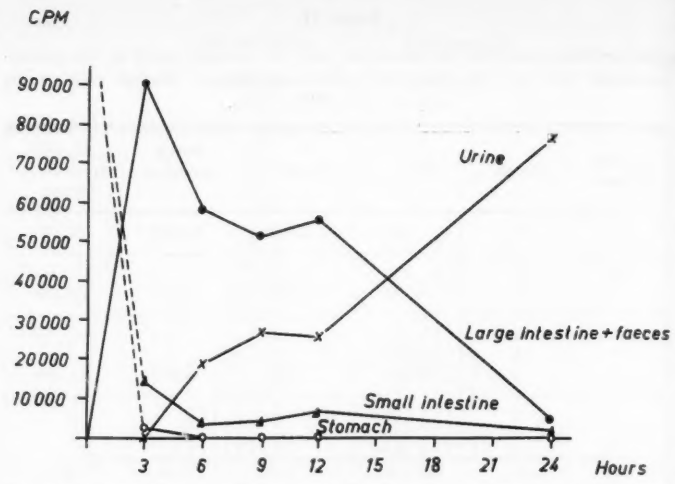


Fig. 2. Radioactivity in different parts of the gastrointestinal tract, faeces and urine after oral administration of labelled chondroitin sulfate to rats, plotted against time.

CPM) each of chondroitin sulfate per os and the excretion was followed. The animals were killed after 3, 6, 9, 12 and 24 hours, respectively, and the radioactivities of the stomach, small intestine and large intestine (plus faeces) were measured separately. The results are shown in table II.

For greater surveyability the sums of these data are plotted in Fig. 2. It is seen that the radioactivity in the urine did not appear until at least 3 hours had elapsed, *i. e.* when the chondroitin sulfate was well down in the large intestine, and the stomach and small intestine were nearly empty. The longer the storage of the chondroitin sulfate in the large intestine, the more radioactivity appeared in the urine. We know that once chondroitin sulfate or inorganic sulfate has entered the blood stream, it is excreted in the urine almost instantly (see below), and therefore radioactive material could not possibly have been absorbed from the small intestine. Thus we must conclude that it was absorbed from the large intestine only.

This sulfate-splitting in the large intestine strongly suggests participation of intestinal bacteria. As a matter of fact, bacterial sulfatases attacking chondroitin and mucoitin sulfates are well known and were first described by NEUBERG and HOFMANN (1931).

**Table III.**

*Radioactivity in the urine and faeces of rats treated with antibiotics and given labelled chondroitin sulfate (130,000 CPM) per os.*

| Rat no. | Hours | Urine CPM | Faeces CPM |
|---|---|---|---|
| 31 | 3 | 60 | — |
| | 6 | 40 | — |
| | 9 | 30 | — |
| | 24 | 10 | 89,300 |
| | 48 | 80 | 13,200 |
| | 72 | 0 | 10,100 |
| | 96 | — | 7,100 |
| | | 220 | 119,700 |
| 32 | 3 | 80 | — |
| | 6 | 60 | — |
| | 9 | 30 | — |
| | 24 | 100 | 75,500 |
| | 48 | 270 | 10,900 |
| | 72 | 0 | 15,600 |
| | 96 | — | 6,600 |
| | | 540 | 108,600 |

To check whether the bacteria in the rat intestine have any sulfatase action, rat faeces were suspended in Krebs' solution, labelled chondroitin sulfate was added and the mixture was incubated overnight at 38° C. 26 per cent of the $S^{35}$ was split off and appeared as inorganic sulfate.

That this bacterial sulfatase activity actually occurs in the lumen of the large intestine in vivo was shown by the following experiment. Rats with bladder fistulas were treated with large doses of antibiotics to depress the bacterial flora in the large intestine, and were later given chondroitin sulfate per os. Urine and faeces were analyzed for radioactivity, and the results are given in table III. Hardly any $S^{35}$ appeared in the urine, almost all being excreted in the faeces. This clearly shows that the bacteria in the large intestine are responsible for the sulfate-splitting action on chondroitin sulfate given per os, and that no intact chondroitin sulfate can be absorbed through the intestinal wall.

Probably the whole chondroitin sulfate molecule is attacked by the bacteria in the gut, and not only the sulfate groups released. In the experiments reported in table I, a small fraction of the radioactivity in the urine did not represent inorganic

**Table IV.**

*Radioactivity in the urine and faeces of rats given 30 mg of inorganic labelled sodium sulfate (168,000 CPM).*

| Rat no. | Hours | Urine CPM | Faeces CPM |
|---|---|---|---|
| 35 | 3 | 100 | — |
| | 6 | 105,000 | — |
| | 9 | 12,900 | — |
| | 24 | 8,500 | — |
| | 48 | 2,300 | 2,400 |
| | 72 | 700 | 3,700 |
| | | 129,500 | 6,100 |
| 36 | 3 | 1,700 | — |
| | 6 | 105,000 | — |
| | 9 | 19,500 | — |
| | 24 | 3,600 | — |
| | 48 | 5,500 | 1,200 |
| | 72 | 200 | 2,700 |
| | | 135,500 | 3,900 |

sulfate but migrated like chondroitin sulfate on the paper electrophoresis strips. Possibly this portion consisted of degradation products of chondroitin sulfate, which, in contrast to high molecular chondroitin sulfate, could be absorbed from the large intestine. Another possibility is that the fraction in question consisted of ethereal sulfates. When radioactive inorganic sulfate is administered intraperitoneally to rats, 5—6 per cent is excreted as ethereal sulfates with the urine within 72 hours (LAIDLAW and YOUNG 1953).

The sulfate ion is reputedly only slightly absorbed from the gastro-intestinal tract (GOODMAN and GILMAN 1955) and sodium sulfate has been widely used as a saline cathartic. On the other hand, the $S^{35}$ (assumingly as inorganic sulfate) released from the chondroitin sulfate in the large intestine seemed to be readily absorbed. Because of this possible discrepancy, an experiment was carried out on the absorption of inorganic sulfate. Inorganic labelled sulfate together with about 30 mg of unlabelled sodium sulfate was administered per os to each rat under brief ether anaesthesia. The radioactivities of the urine and the faeces were determined at the usual intervals and the results are shown in table IV.

**Table V.**

*Recovery of $S^{35}$ in pooled urine and faeces after subcutaneous administration of chondroitin ($S^{35}$) sulfate to six rats.*

| Amount of chondroitin sulfate administered | Number of rats | Hours | Urine | | | Faeces CPM |
|---|---|---|---|---|---|---|
| | | | Total radioactivity CPM | Per cent dialyzable radioactivity | Per cent $S^{35}$ as inorganic sulfate | |
| 150 mg 3,522,000 CPM | 6 | 6 | 1,040,400 | 4.1 | 1.8 | 0 |
| | | 18 | 1,077,300 | 19.3 | 15.2 | 51,100 |
| | | 42 | 365,700 | 59.8 | 52.8 | 77,100 |
| | | 66 | 68,500 | 69.0 | — | 57,400 |
| | | 90 | 36,500 | — | — | 43,400 |
| | | 162 | 58,300 | — | — | 13,300 |
| | | | 2,646,700 (75.2 %) | | | 242,300 (6.9 %) |

The sulfate ions proved to be surprisingly well absorbed from the gut as most of the radioactivity was excreted with the urine. However, as after oral administration of chondroitin sulfate, very little of the $S^{35}$ appeared in the urine within the first three hours. This possibly means that the sulfate ions were not absorbed from the small intestine, but from the large intestine only.

The amount of sodium sulfate absorbed in this experiment represents more sulfate ions than could have been released from the chondroitin sulfate given per os (table I). Thus, it is quite possible that the sulfate group of chondroitin sulfate is absorbed from the large intestine as inorganic sulfate.

The results of the experiments with subcutaneous administration of labelled chondroitin sulfate are given in tables V and VI. $S^{35}$ rapidly appeared in the urine, and most of the radioactivity (60—82 per cent) was excreted by the kidneys already in 18 hours. After that the radioactivity in the urine dropped abruptly, although traces of $S^{35}$ would persist for weeks.

At first most of the labelled material came out in a high molecular form, most probably intact chondroitin sulfate, which could not pass through the dialysis membrane. But later the dialyzable fraction in the urine increased with a parallel increase of the amount of $S^{35}$ as inorganic sulfate as determined after separation by paper electrophoresis. Obviously most of the dialyzable material consisted of inorganic sulfate which had been split off from the mucopolysaccharide molecule. In the 42-hour portions

**Table VI.**

*Recovery of $S^{35}$ in the urine, bile and faeces after subcutaneous administration of chondroitin ($S^{35}$) sulfate to rats with bile and bladder fistulas.*

| Amount of chondroitin sulfate administered | Number of rats | Hours | Urine | | Bile CPM | Faeces CPM |
|---|---|---|---|---|---|---|
| | | | Total radio-activity CPM | Per cent $S^{35}$ as in-organic sulfate | | |
| 100 mg 1,500,000 CPM | 1 | 6 | 746,400 | 1.3 | 1,150 | 600 |
| | | 18 | 458,500 | 7.5 | 2,900 | 1,800 |
| | | 42 | 132,000 | 53.9 | 3,700 | 4,600 |
| | | 66 | 12,600 | — | 250 | — |
| | | | 1,349,500 (90.0 %) | | 8,000 (0.5 %) | 7,000 (0.5 %) |
| 50 mg 776,000 CPM | 1 | 6 | 351,000 | 4.1 | 100 | 200 |
| | | 18 | 288,000 | 18.3 | 2,200 | 300 |
| | | 42 | 77,800 | — | 1,700 | 1,400 |
| | | 66 | 4,200 | — | 1,700 | 800 |
| | | | 721,000 (92.9 %) | | 5,700 (0.7 %) | 2,700 (0.3 %) |

more than half of the radioactivity in the urine was ascribable to inorganic sulfate. It was felt that this ratio did increase even further in the following urine samples, but low activities in the urine made the separation and determination of sulfate unreliable.

A small fraction (less than 1 per cent) of the $S^{35}$ administered was found in the bile in two experiments (table VI).

In table V the activities found for the faeces were higher than in table VI. The latter experiments, however, must be considered more reliable because in these the urine was collected through bladder fistulas. This arrangement made the contamination of faeces with urine impossible and increased the total recovery of radioactivity.

Bacterial depolymerization of hyaluronic acid can occur even in the presence of toluene, as shown by JENSEN (1955). To rule out the possibility that the free sulfate, found in the urine in several experiments, might have been split off by bacteria or urinary enzymes instead of in the body of the rat, the following experiment was carried out. 20 mg chondroitin sulfate were dissolved in 5 ml of distilled water in a flask. Toluene was added and urine from a normal rat was collected in this flask for 60

**Table VII.**

*Radioactivity in the urine and faeces of rats treated with antibiotics and given labelled chondroitin sulfate subcutaneously.*

| Amount of chondroitin sulfate administered | Number of rats | Hours | Urine | | Faeces CPM |
|---|---|---|---|---|---|
| | | | Total radio-activity CPM | Per cent $S^{35}$ as inorganic sulfate | |
| 100 mg 8,660,000 CPM | 1 | 6 | 3,380,000 | 2.2 | 0 |
| | | 18 | 3,040,000 | 5.4 | 8,000 |
| | | 42 | 350,000 | 22.2 | 130,000 |
| | | 66 | — | — | 85,000 |
| | | | 6,770,000 (78.2 %) | | 223,000 (2.6 %) |
| 100 mg 8,660,000 CPM | 1 | 6 | 1,160,000 | 0.2 | 0 |
| | | 18 | 3,355,000 | 1.3 | 10,000 |
| | | 42 | 2,830,000 | 11.1 | 70,000 |
| | | 66 | 210,000 | — | — |
| | | | 7,555,000 (87.2 %) | | 80,000 (0.9 %) |

hours at room temperature. On subsequent dialysis, 6.8 per cent of the chondroitin sulfate proved to have become dialyzable, whereas normally only 0.25 per cent was dialyzable. Paper electrophoresis runs showed, however, that the dialyzable material migrated like chondroitin sulfate and not like inorganic sulfate. The urine probably has some depolymerizing properties versus chondroitin sulfate but no sulfatase action, and the free radioactive sulfate found in the urine must have passed the kidneys in that form.

There are still at least two possibilities as to the site of the sulfate-splitting after subcutaneous administration. It might have been caused by a sulfatase in the tissues of the rat, or it might have occurred in the large intestine as a result of bacterial activity there. From the tables V and VI it is evident that $S^{35}$ could reach the gastro-intestinal tract after subcutaneous injection of labelled chondroitin sulfate. If this radioactivity represented chondroitin sulfate, it ought to have been destroyed in the large intestine like the chondroitin sulfate fed per os (cf. above). In such case much of the $S^{35}$ would have been reabsorbed as inorganic sulfate, and it might have formed all the inorganic sulfate in the urine.

In order to differentiate between these two possibilities, rats were treated with antibiotics as in previous experiments, and given labelled chondroitin sulfate subcutaneously. The doses of the antibiotics were large enough to depress bacterial sulfatase activity in the intestine almost completely (cf. table III). The result of this experiment is shown in table VII. In spite of the antibiotics, the urine contained inorganic sulfate, and the concentration of this compound (per cent of total activity) increased during the experiment (cf. tables V and VI). These results indicate that the sulfate group of chondroitin sulfate can be split off in the tissues of the rat and not only in the lumen of the large intestine by the influence of bacteria.

*In vitro* experiments with incubation of homogenates from various organs and connective tissues, including the cornea, to which labelled chondroitin sulfate had been added, were all negative in regard to sulfatase action. Samples were taken out during a period of time and dialyzed, but no inorganic labelled sulfate could be detected in the dialysates in spite of the sensitivity of the method; sulfate split off from 0.025 mg of chondroitin sulfate would easily have been detected.

In another experiment $S^{35}$ was introduced into the chondroitin sulfate of living rats according to BOSTRÖM (1952). The animals were killed after 5 days and the costal cartilage was taken out (it was then practically free from inorganic radioactive sulfate (BOSTRÖM 1952). Fresh and boiled slices of the cartilage were incubated separately in Krebs' phosphate buffer pH 7.4 together with some extra unlabelled sulfate. If any sulfatase had exerted its action within the unboiled cartilage slices, some ($S^{35}$) sulfate ions would have been released into the solution. However, none were found.

There are obvious discrepancies between the *in vivo* and the *in vitro* experiments. The *in vivo* results strongly suggest the existence of a sulfatase attacking chondroitin sulfate, as probably no simple ion exchange occurs between the inorganic sulfate in the tissues and the sulfate groups of chondroitin sulfate (BOSTRÖM and MÅNSSON 1952). Possibly such an enzyme is present in the subcutaneous tissue, since chondroitin sulfate deposited there will lose sulfate, the loss increasing with the retention of the chondroitin sulfate. The fact that no sulfatase action could be demonstrated in the *in vitro* experiments might only indicate that the enzyme is very fragile and did not survive

the experimental procedures. More *in vitro* work is obviously needed before the existence of a chondrosulfatase can be regarded as definitely proved.

## Summary.

When cartilage chondroitin ($S^{35}$) sulfate was administered subcutaneously to rats, 60 to 82 per cent of the radioactivity was excreted in the urine already within 18 hours. Less than one per cent appeared in the bile during the whole experiment and never more than a few per cent were excreted in the faeces.

Although most of the chondroitin sulfate was eliminated as such, some of the $S^{35}$ in the urine appeared in the form of inorganic sulfate, and the ratio of inorganic sulfate to chondroitin sulfate increased during the course of the experiment. This suggested the existence of a sulfatase attacking chondroitin sulfate.

However, *in vitro* incubation of homogenates from various tissues together with labelled chondroitin sulfate did not reveal any sulfatase activity.

Radioactive chondroitin sulfate given per os to rats was not absorbed in intact form from the gastrointestinal tract. It was, however, attacked by the bacteria in the large intestine, and most of the radioactivity was absorbed as inorganic sulfate and excreted as such in the urine.

Sulfate ions in fairly large amounts were well absorbed from the intestine and probably from the large intestine only.

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# Release of Adenosine Triphosphate along with Adrenaline and Noradrenaline Following Stimulation of the Adrenal Medulla.

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Large amounts of adenosine triphosphate (ATP) have been detected in the adrenal medullary granules containing adrenaline and noradrenaline (HILLARP, HÖGBERG and NILSSON 1955, FALCK, HILLARP and HÖGBERG 1956). The experiments reported below were done in order to investigate the possible rôle of ATP in the storage and release of catechol amines.

## Experimental.

The adrenal medulla was stimulated *via* the splanchnic nerves using two procedures (*cf.* VOGT 1954), namely by injection of morphine to cats and by insulin treatment of rats.

Twelve cats weighing about 2.5 kg were divided into two groups of six animals. One group served as control. The other group received a subcutaneous injection of morphine hydrochloride in a dose of 20 mg per kg body weight. Depending on the degree of excitation produced some of the animals received another dose of 10 or 20 mg per kg body weight two hours later. After another two hours the animals were killed. The adrenals were dissected out, chilled, freed of adhering tissue, and weighed. The adrenal medullas from all the twelve glands in either group were dissected out, pooled and weighed. Care was taken to keep the glands ice-cooled throughout the preparations. The pooled medullas from either group were transferred to a centrifuge tube, crushed by means of a glass rod, and extracted repeatedly with 0.4 N perchloric acid (total volume 9 ml). After centrifugation the super-

**Table I.**

*Catechol Amines and ATP in Adrenal Medullas of Cats Following Stimulation by Means of Injections of Morphine.*

| | Morphine treated | Controls | Per cent difference |
|---|---|---|---|
| Weight of pooled medullas[1], mg .. | 288 | 248 | — |
| Catechol amines, mg per g medulla | 2.41 | 8.40 | — 71 |
| ATP, mg per g medulla | | | |
| a) computed from acid labile P . | 1.23 | 3.91 | — 69 |
| b) computed from ultraviolet absorption ..... | 1.24 | 4.28 | — 71 |

[1] Both medullas of six cats.

natants were neutralized. Aliquots were assayed for catechol amines according to EULER and HAMBERG (1949) and for inorganic and acid labile P (8 min. hydrolysis in N HCl at 100° C) according to FISKE and SUBBAROW (LE PAGE 1951). A third aliquot from either sample was used for estimation of adenine compounds by measuring ultraviolet absorption. The samples were first passed through a cation exchange column (Dowex 50, hydrogen form, 150—300 mesh, 3 cm × 1 cm²) in order to remove catechols and other interfering material. The adenosine phosphates were then precipitated quantitatively by adding 10 per cent $ZnSO_4$, 7 $H_2O$ and adjusting pH to about 6 by means of 0.1 N NaOH. The precipitate was washed once with $H_2O$ and dissolved in N $NH_4OH$. The solution was transferred to an anion exchange column (Dowex 2, chloride form, 200—400 mesh, 1.8 cm × 0.55 cm²). Elution was performed at a rate of 0.2—0.3 ml per minute according to the following scheme (cf. COHN and CARTER 1950): 7 × 10 ml of 0.003 M HCl, 7 × 10 ml of 0.02 M NaCl in 0.01 M HCl solution, 5 × 10 ml of 0.2 M NaCl in 0.01 M HCl solution. This procedure was used in order to remove interfering substances rather than fractionate the adenosine phosphates, which may have been partly hydrolyzed owing to the high pH of the dissolved zinc precipitate. The various fractions were read in a Beckman Spectrophotometer model DU in the ultraviolet region, and almost pure adenine spectra with peaks at 257 mμ were obtained. Table I gives the sum of the AMP, ADP and ATP recovered, computed as ATP. The figures thus computed were in good agreement with the ATP figures computed from the acid labile P, suggesting that the major part of adenine and acid labile P, respectively, are present as ATP, which is in accordance with earlier findings (FALCK *et al.*, *l. c.*).

In a second experiment 30 male adult rats (200—300 g) were divided into two groups of 15 animals, which had been starved for 24 hours before the experiment. One group served as control, while the other group was treated with a single subcutaneous injection of insulin (Vitrum) in a dose of 10 I.U. per 100 g body weight. Care was taken to prevent the animals from getting severe convulsions and asphyxia

**Table II.**

*Catechol Amines and Acid Labile P in Adrenal Medullas of Rats Following Stimulation by Insulin Injections.*

| | Insulin treated | Controls | Per cent difference |
|---|---|---|---|
| Weight of pooled medullas[1], mg .. | 52.6 | 52.7 | — |
| Catechol amines | | | |
| Total, mg per g medulla ..... | 3.12 | 10.8 | — 71 |
| Percentage distribution: | | | |
| fraction S[2] ..... | 10 | 9 | |
| fraction G[3] ..... | 76 | 79 | |
| fraction A[4] ..... | 14 | 12 | |
| Inorganic P, mg per g medulla | | | |
| fraction G[3] ..... | 0.094 | 0.061 | + 54 |
| fraction A[4] ..... | 0.452 | 0.460 | — 2 |
| Acid labile P, mg per g medulla | | | |
| fraction G[3] ..... | 0.228 | 0.805 | — 72 |
| Protein, mg N per g medulla | | | |
| fraction G[3] ..... | 6.84 | 7.78 | — 12 |

[1] Both medullas of 15 rats.
[2] Obtained by centrifugation of homogenate at 800 × *g* for 4 minutes.
[3] Obtained by centrifugation at 6,800 × *g* for 60 minutes ("granules").
[4] Supernatant ("extragranular cytoplasm").

by injecting 2 ml of a 5 per cent solution of glucose subcutaneously at the first sign of convulsive movements. Five hours after the injection of insulin the animals were killed. The adrenal medullas from either group were dissected out, pooled, weighed, and homogenized in 4 ml 0.3 M ice-cooled sucrose as described in a previous paper (HILLARP, LAGERSTEDT and NILSON 1953). Fractional centrifugation was then performed as described earlier (FALCK *et al.*, *l. c.*). The granules and the supernant were analyzed for catechol amines and inorganic and acid labile P (*vide supra*). The total adenine content was also estimated by reading the ultraviolet absorption. In this case, however, fractionation by means of anion exchange was omitted, and, consequently, the adenine spectra obtained were not pure enough to permit an accurate estimation.

Total granular protein was estimated on the precipitate formed after adding perchloric acid to the granular suspension, according to CLELAND and SLATER (1953).

## Results.

Tables I and II show the results from the cat and rat experiments, respectively. Both the catechol amines and the acid labile P dropped to about 30 per cent of the control values following

stimulation with morphine and insulin, respectively, the proportions between the remaining catechol amines and acid labile P thus staying unchanged. Judging from the UV absorption data (Table I) there was apparently a drop also in the adenine moiety of the adenosine triphosphate indicating a release of the entire molecule along with the catechol amines. The possibility still exists, though, that the molecule was split before being released.

The total granular protein dropped 12 per cent in the treated rat group (Table II), which may not be significant, in view of a possible slight contamination with extramedullar granules, which may have occurred to a different extent in the two preparations.

## Discussion.

The finding that the catechol amines and the ATP disappear in the same proportions during stimulation of the adrenal medulla strongly argues for a specific rôle of the latter compound in the storage and release of the former. So far, however, very little can be said about the mechanism involved. Preliminary studies of these laboratories show that the granules have a high adenosine triphosphatase activity, from which the adenosine triphosphate of the unimpaired granules are protected in an unknown way, although ATP added to the suspension medium is attacked by the enzyme. It is tempting to speculate, then, that enzymatic splitting of ATP is involved in some way or other in the release mechanism; perhaps the enzyme is enabled to act on the ATP inside the granules.

The fact that the granular protein stayed almost unchanged during stimulation will exclude the possibility of a secretion of whole granules, in agreement with earlier histologic observations by HILLARP, HÖKFELT and NILSON (1954). Furthermore, since the soluble protein of the granules constitute a little more than half of the total granular protein, the drop in granular protein is much less than would have been expected (35—40 per cent), had the entire soluble protein fraction been released during stimulation. A profound damage of the granular membranes of the stimulated medullas therefore seems unlikely. Most probably the stimulatory mechanism is specifically directed towards the release of the ATP and the catechol amines, the granular structure staying essentially intact.

It is interesting to note the absence of an accumulation of either inorganic phosphate or catechol amines in the extragranular cytoplasm of the stimulated medullas. Apparently the substances released from the granules have little difficulty in permeating the cellular membrane. (It should perhaps be mentioned that any accumulation of ATP in the extragranular cytoplasm would result in an increase in inorganic P owing to splitting of ATP during the preparation, if not earlier.)

## Summary.

The adrenal medullas of cats and rats were stimulated *via* the splanchnic nerves by means of injections of morphine and insulin, respectively.

In both types of experiment stimulation resulted in a drop in the ATP content of the adrenal medulla, the percentage decrease being the same as for the catechol amine content.

During stimulation ATP and the catechol amines are released from the adrenal medullary granules, which remain in the medullary cells, probably essentially intact.

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# Determination of Carbon Monoxide in Small Gas Volumes.

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The determination of carbon monoxide can be carried out by several different methods (cf. ALTERI 1945). Chemical and physical methods have been used. Of the latter, the hopcalite method is sensitive and convenient, though not absolutely specific. This non-specificity is generally of no practical significance in physiological work (cf. SIÖSTEEN and SJÖSTRAND 1951). LINDELÖV and SJÖSTRAND constructed a hopcalite apparatus (SJÖSTRAND 1944 and 1948) for the measurement of very low CO concentrations. However, this, as well as other hopcalite apparatuses, requires relatively large volumes of gas for an analysis. The gas to be analysed passes at a constant rate of about 1 litre per min. through the apparatus. CO is catalytically burnt in a combustion chamber. The heat evolved is measured by means of a differential thermometer. After about 6 minutes, heat equilibrium is approached, *i. e.* the heat lost from the combustion chamber equals that evolved by the combustion of CO.

In connection with blood analyses or respiratory physiological studies, it is an advantage to be able to perform CO determinations also with small gas volumes. If the total amount of CO to be analysed were introduced into the combustion chamber during a short period of time, a higher temperature might be expected

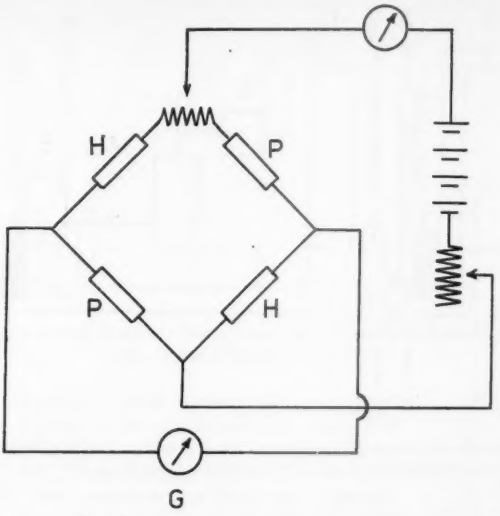


Fig. 1. Circuit of the bridge for temperature measurements. The Wheatstone bridge arms consist of thermistors, two of them (H) in the hopcalite part and the other two (P) in the pumice part of the measuring cell. A direct writing potentiometer may be substituted for the galvanometer, G.

in the combustion chamber than if the same total amount of CO of a low concentration were made to pass through the apparatus during a longer period. With the forementioned combustion period, the sensitivity of the apparatus to a certain amount of CO might therefore be expected to increase.

This paper deals with a method of CO analysis according to the above-mentioned principles. In connection with the present investigation, a new hopcalite apparatus (AB Stålex, Stockholm) is used in which electrical temperature measurement affords convenient registration on direct writing instruments.

## Methods.

*CO-meters.* In the beginning of this work the Lindelöv-Sjöstrand CO-meter was used. Later on the measurements were made on the new Stålex hopcalite apparatus in which a thermistor Wheatstone's bridge was substituted for the differential thermometer of the old apparatus (see Fig. 1). In this way the sensitivity was increased and the changes in temperature could be read on a galvanometer or conveniently be obtained with a direct writing potentiometer. A Philips direct writer,

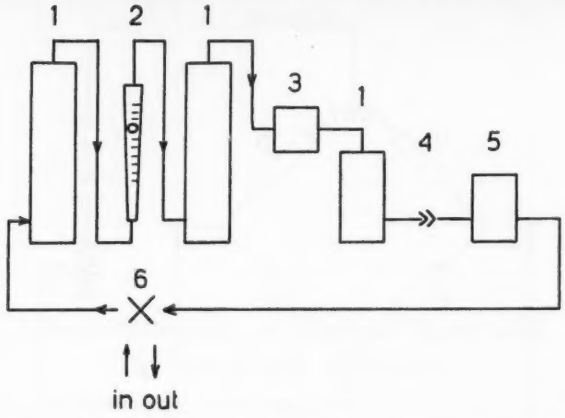


Fig. 2. Scheme of the gas flow through the CO-meter. 1 KOH filter, 2 gas flow meter, 3 measuring cell, 4 gas flow regulator, 5 pump, 6 four way stop-cock.

type PR 2200 A/21, with a sensitivity of 2.5 or 5 mV for a whole scale deflection was used. Another important improvement is a sensitive gas flow meter (rotameter), necessary to check the constancy of gas flow through the meter. A scheme of the gas flow through the apparatus is given in Fig. 2.

Gas first passes two filters with KOH (1) and the rotameter (2) between them, then through the measuring cell (3), containing pumice in one half and hopcalite in the other. It flows further through one more KOH filter (1), (inserted to prevent moisture from reaching the cell through the gas exit tube), and finally through a needle flow regulator (4) which adjusts the gas flow produced by the pump (5) of the CO-meter. By means of a 4 way stop-cock gas may be directed in a closed circuit in the apparatus to obtain a zero-value. By turning the stop-cock gas samples may be sucked into the meter.

In this investigation the pump of the apparatus was excluded and an external pump was used to suck gas through the CO-meter. In this way a smoother and more constant flow of gas was obtained. Usually air (in some experiments 100 per cent $O_2$) was sucked continuously through the meter. In order to get CO free air into the CO-meter an external hopcalite filter was used after a KOH filter, see Fig. 3. Gas samples to be analysed were introduced behind the hopcalite filter by means of a 3 way stop-cock from a rubber bag or were injected by means of a syringe through a rubber membrane. In front of the CO-meter there were two more filter bottles which were necessary to get reproducible deflections when highly concentrated CO samples were injected.

During the first part of the investigation the external pump was a vacuum oil pump with a pressure equilibration chamber and two

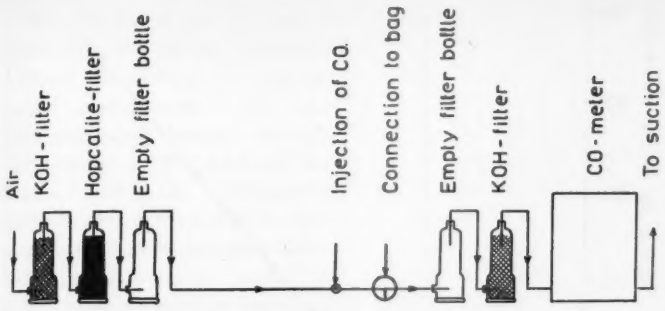


Fig. 3. Filters added using air flow through the CO-meter and sites for introduction of gas samples for analysis.

pressure reduction valves between the pump and the meter. Later on an ejector suction pump (IR 1860, Aga, Stockholm), driven by compressed air was used, and in combination with the needle flow regulator a very constant gas flow was obtained.

*Measurements of the volumes* of the gas samples introduced into the CO-meter was made in several ways.

1. By an injection syringe.

2. By sucking gas into the meter with a constant flow rate for a known time from a rubber bag. The rotameter reading was calibrated with a water gas meter (Nordgas).

3. By means of a four way stop-cock (Fig. 4).

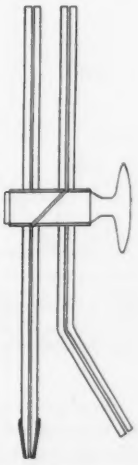

Fig. 4. Four way stop-cock used to measure small volumes of CO.

The last mentioned method requires some comment. It is used for measurements of small gas volumes. The gas to be measured is allowed to flow through the bore of the four way stop-cock to fill it completely. The bore of the two cocks used, had a volume of 0.02 and 0.07 ml. They were weighed out with mercury. After the gas flow through the cock is stopped, it is turned and the gas volume in the bore of the cock is sucked down into a recipient over mercury. CO free gas from a rubber bag is used to wash out the stop-cock bore completely. About 20 ml of CO free gas sufficed for this. From the recipient the gas is injected into the CO-meter.

The stop-cock method was especially adapted for measuring small volumes of concentrated CO (100 per cent) for comparison with small amount of CO driven out of blood samples (LINDERHOLM, SJÖSTRAND and SÖDERSTRÖM 1956), but it was found useful also for the determination of the absolute CO concentration in diluted gas mixtures and for calibration.

Fig. 5. Scale deflexions in relation to the volume of two CO gas mixtures, one with 0.118, the other with 0.058 per cent CO in air, injected with a syringe during about 5 sec. Each point is the mean of 10 determinations. The size of the dots covers $\pm$ 3 times the standard error of the mean.

*Production of pure CO for calibration* was made essentially according to the method described by HALLBERG (1955). Formic acid (proanalysi, Merck) was heated in water to 88° C. Concentrated sulphuric acid was dropped into the formic acid and the gas evolved was passed through a washing bottle with 10 % NaOH and through a filter bottle with KOH or NaOH in substance to the four way stop-cock for measurement of small volumes. Rubber tubings have been avoided as far as possible. A short vacuum rubber tube connected the filter bottle with one way of the four way stop-cock.

## Results.

The two CO-meters used behaved essentially alike. The results obtained during the development of the method for analysis of CO in small gas volumes, are illustrated by the following experiments.

*Rapid introduction of various amounts of CO into the CO-meter.* Various amounts of CO were injected during about 5 sec. into the gas (air) flowing through the apparatus. The maximal deflection on the differential thermometer of the Lindelöv-Sjöstrand-meter or the potentiometer writer in connection with the Stålex-meter was then found to be proportional to the amount of CO injected, see Fig. 5. The deflection in relation to time after a rapid injection of CO is shown by Fig. 6 (which also shows the deflection when a CO gas mixture is sucked through the meter for six minutes, see below). The optimal flow rate of gas through the CO-meter varied with the type of apparatus. Fig. 7 shows the variation in maximal deflection with rate of gas flow after the rapid injection of a constant amount of CO.

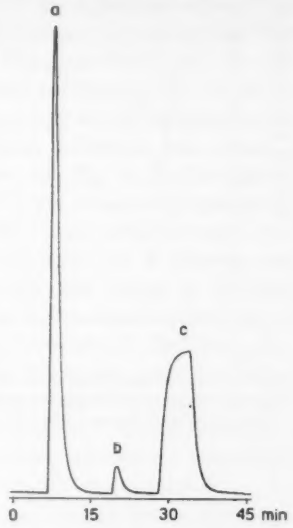


Fig. 6. Deflections obtained on the direct writing potentiometer a: after instantaneous injection of CO; b: after suction of a CO gas mixture for 20 seconds through the CO-meter; c: after suction of the same gas mixture used at b during 6 minutes, the total amount of CO introduced into the CO-meter in this case being equal to that introduced instantaneously at a.

*Introduction of the same total amount of CO during various lengths of time.* With a syringe the same amount of gas was injected evenly but at different rates. The magnitude of the deflections was the same when the injection was completed within 20 sec. If the injection lasted for a longer time the deflections decreased.

*Introduction of a CO gas mixture of certain concentration with constant rate during various length of time.* By turning a 3 way stopcock by hand or by using an electro-magnetic 3 way valve and an electric timing watch, either CO free air or CO air mixture from a rubber bag could be sucked through the CO-meter. If the time for the suction of the CO mixture was varied the deflections of the meter were found to be proportional to the time, *i. e.* to the amount of CO sucked through the apparatus, as long as this time period was less than 20 seconds. If the CO mixture

Fig. 7. Units of scale deflections for instantaneous injections of the same amount of gas at various flow rates through the CO-meter. Each point is the mean of 5 determinations. Vertical lines are ± 3 times the standard error of the mean.

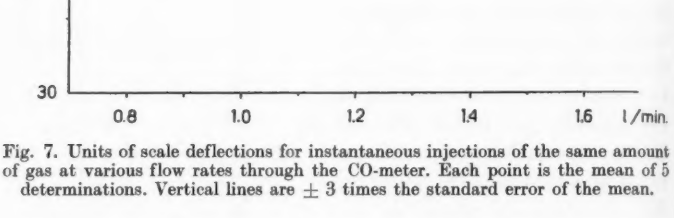


Fig. 8. Units of scale deflection in relation to the time of suction of a gas mixture through the CO-meter. For details cf. Fig. 7.

was sucked through the meter for a longer time the deflection was relatively smaller in relation to the total amount of gas administered (Fig. 8). The greatest deflections were obtained at high flow rates, up to 2 litres per min., through the CO-meter.

*Comparison between the magnitude of the deflections when CO is introduced at a constant rate during 6 minutes and during less than 20 seconds.* The same total amount was introduced into the CO-meter: 1) during 20 seconds, 2) at a constant rate during 6 minutes. In the first case, to some degree depending on the apparatus and the flow rate through it, the maximal deflection was about 3 times greater than in the second case, see Fig. 6. In this second case the deflection reaches a steady state which is proportional to the CO concentration of the gas. When gas with the same concentration was sucked through the CO-meter for 6 minutes and for 20 seconds the maximal deflection was about 4—5 times greater in the first case. This ratio also varies slightly with various apparatuses and with the rate of gas flow through the apparatus.

*Calibration of the measurements.* The hopcalite method requires repeated calibrations as the sensitivity of the CO-meter changes with a gradual slow decrease of the activity of the hopcalite.[1] A standard amount of CO was therefore introduced frequently into the meter, usually before each sample was analysed.

The absolute amount of CO in a gas sample was determined by comparing the deflection obtained with the sample and that obtained with a known amount of CO when each of them are introduced into the CO meter in less than 20 seconds. In routine analyses CO is most easily obtained from a steel bottle. Commercially available compressed CO tanks do not contain 100 per cent CO and as the CO concentration may vary, it must be determined for each tank. Comparisons were made between tank gas and CO produced from formic acid (see under methods). The latter is in all probability very nearly 100 per cent CO gas. The gas volumes used were measured with the four way stop-cock, sucked down into a recipient as described under methods and flushed into the CO-meter. The results of comparisons between concentrated CO in a tank and CO produced from formic acid are shown in Table I. The recipient also allowed the measurement and introduction of 100 ml of a CO gas mixture of low concentration. For instance, 0.01 per cent CO may thus with fair accuracy be directly compared with the 100 per cent gas and errors connected with dilution procedures are avoided.

*The influence of the gas mixture used for the continuous flow*

[1] In spite of this decrease in activity the same hopcalite may be used for about one week. During this period the deflections for a standard amount of CO should not decrease by more than 10—20 per cent. The hopcalite must then be regenerated by heating to 150° C for 12 hours.

*through the CO-meter on the deflection for a known amount of CO.* Any gas mixture differing from the gas flowing continuously through the CO-meter may cause small unspecific deflections of the CO-meter, especially if large volumes are introduced. It is therefore desirable that the gas mixture flowing through the CO-meter has the same composition as the gas mixture in which the CO sample is diluted.

It seemed to be of special interest to test if a certain amount of CO in 100 per cent $O_2$ gave the same deflection of the CO-meter as the same amount of CO in air when there was a continuous flow of air or oxygen through the CO-meter. If the volume of the gas sample was less than 100 ml of CO in $O_2$ or air the same deflections were obtained when air was used for continuous flow. If the volume of the gas samples was increased CO in $O_2$ gave slightly bigger deflections than the same amount of CO in air; the difference in a series of determinations being $1.9 \pm 0.62$ per cent ($n = 14$) for gas sample volumes of 200 ml and $4.3 \pm 1.3$ per cent ($n = 14$) for volumes of 450 ml. Thus corrections should be applied when measurements are made on CO — $O_2$ samples above 100 ml. When $O_2$ was used for the continuous flow through the meter the deflections for a standard amount of CO were usually a few per cent higher than when air was sucked through the meter and a certain amount of CO contained in $O_2$ caused a few per cent larger deflections than when it was diluted in air even when the volume of gas sample was rather small.

*Sensitivity and error of the method.* The maximal sensitivity varied with the different apparatuses and with the quality of the hopcalite. The Stålex CO-meter with its built-in microamperemeter had a sensitivity of the same order as the old Lindelöv-Sjöstrand apparatus. With an external galvanometer (Multiflex G 3) full scale deflections were obtained with 0.01—0.02 ml of CO at instantaneous injection and with a CO concentration of about 1 part in $10^5$ after 6 minutes flow through the CO-meter. An amount of 1 $\mu$l of CO injected within 20 seconds and a concentration of about 1 part CO in $10^7$ parts of air or oxygen with the "steady state method" can be determined. In the last part of the investigation the Stålex CO-meter was usually combined with a potentiometer writer (Philips PR 220/A21), giving a full scale deflection for 2.5 mV. With this arrangement the sensitivity was the same as with the Multiflex galvanometer.

**Table I.**

*Comparison between CO from a steel bottle and CO produced from formic acid. Small volumes of CO were measured with the four way stop-cock (see text). $M_1$ and $M_2$ are mean scale deflection units. Each pair of $M_1$ and $M_2$ represents analysis during one day. $\varepsilon M$ is the standard error of the mean.*

| CO from steel bottle | | | CO produced from formic acid | | | |
|---|---|---|---|---|---|---|
| n | $M_1$ | $\varepsilon M_1$ | n | $M_2$ | $\varepsilon M_2$ | $\frac{M_1}{M_2} \cdot 100$ |
| 5 | 11.01 | 0.020 | 9 | 11.34 | 0.023 | 97.09 |
| 9 | 11.33 | 0.036 | 7 | 11.68 | 0.026 | 97.00 |
| 5 | 10.92 | 0.034 | 5 | 11.25 | 0.028 | 97.07 |
| 8 | 11.33 | 0.019 | 10 | 11.68 | 0.023 | 97.00 |
| 6 | 11.50 | 0.022 | 7 | 11.86 | 0.023 | 96.97 |
| 5 | 11.93 | 0.050 | 6 | 11.57 | 0.038 | 96.98 |
| | | | | | | M . 97.02<br>$\varepsilon$M . 0.020 |

The error of the method when CO is injected with the 4 way stop-cock is demonstrated in Table I. The coefficient of variation for a single determination is usually 0.5—1 per cent. When CO gas mixtures are injected with a syringe or sucked into the CO-meter from a rubber bag a chosen short period of time the coefficient of variation is usually 0.5 to 2 per cent. At small scale deflections, especially when small volumes are injected or when gas is sucked into the CO-meter for only a few seconds and also when big amounts of CO are introduced, the coefficient of variation increased to 2—3 per cent.

## Summary.

A method is described for analysis of CO in small gas volumes with hopcalite CO-meters. By the use of electrical temperature measurement and registration with direct writing potentiometers the applicability of these CO-meters has been greatly increased. Amounts of pure CO, from about 0.001 ml up to about 1 ml, can be measured with marked accuracy in gas volumes from 0.02 to about 500 ml irrespective of the CO concentration. Accurate calibration can be obtained by comparison with 100 per cent

CO without dilution procedures. The method was found especially suitable for analysis of small amounts of CO extracted from blood samples.

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For technical assistance the authors are much indebted to D. HASENSON, Fil. mag.

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# Symmetric Hydrolysis with Intestinal Enzymes, Acid-Neutralizing Effect and Toxicity of Isopropyl Esters of Phenylalanine Isomers.

By

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In preparation of the isomers of phenylalanine, use has been made of asymmetric hydrolysis of the isopropyl ester of DL-phenylalanine with certain pancreatic enzymes (WRETLIND 1950). These enzymes hydrolyze the L ester only, so that separation is easily produced of free, water-soluble L-phenylalanine and the unhydrolyzed, ether-soluble isopropyl ester of D-phenylalanine. In view of these experiments, one would be inclined to assume that only the isopropyl ester of L-phenylalanine could be utilized by the organism as a source of phenylalanine. Whether or not this applied could most easily be ascertained by following the growth in rats given a synthetic diet devoid of phenylalanine, but containing the L and the D ester of phenylalanine, respectively. It was, however, found in such experiments that the isopropyl ester of both L- and D-phenylalanine was able to produce growth in rats under the aforementioned conditions (WRETLIND 1954). A possible explanation is that enzymes are present in the gastrointestinal tract which hydrolyze the DL ester symmetrically.

An account is given in the present paper of experiments concerning the effect on the isopropyl ester of DL-, D- and L-phenylalanine of enzymes from the duodenal mucosa that were found to contain enzymes hydrolyzing both the D and the L ester.

Since this provides a relatively stable basic ester which is readily split in the intestine, its use could be envisaged to produce a neutralizing effect on the hydrochloric acid of the stomach. The ester is then converted into hydrochloride. When this ester hydrochloride enters the intestine, the ester is hydrolyzed and the chloride ions are released at the same time. Neutralization of the hydrochloric acid in the stomach is thus achieved without changing the acid-base balance of the body as is the case, for example, in neutralization of excess gastric acidity by sodium bicarbonate. With this object in mind, a study was made of the acid-neutralizing effect of the ester. The toxicity of the isopropyl esters of phenylalanine isomers was also determined.

## Experimental.

*The isopropyl esters of DL-, D- and L-phenylalanine* were prepared according to WRETLIND's technique (1950, 1954).

*Enzyme preparation.* A freeze-dried extract from the hog duodenal mucosa was used for determination of hydrolysis of the isopropyl ester. The extract was prepared by adding 9 parts of distilled water to 1 part of frozen hog duodenal mucosa; the mixture was then allowed to stand with stirring for 30 minutes at room temperature. After filtration, the filtrate was freeze-dried. About 10 g of dry powder were obtained from 1 liter of filtrate. The dry powder was dissolved in distilled water on the same day that determinations of the hydrolysis were made.

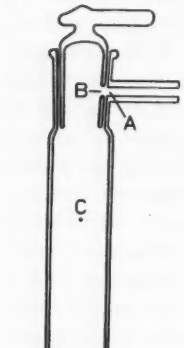


Fig. 1. *Tube used in hydrolysis of the isopropyl esters of phenylalanine.*

*Hydrolysis procedure.* The reaction was performed in tubes provided with a lateral tube beside the ground stopper, which served as a tap for this lateral tube. The appearance of the tube is shown in Fig. 1. When the various solutions had been poured into the tube, the stopper was inserted so that holes A and B were opposite each other. The lateral tube was then connected to a vacuum pump. After a few seconds, the stopper was turned to produce closure of the opening A—B; the tube was then lowered into a water bath in a rotating stand, arranged so that the tube was revolved about point C. The tube was revolved in this way about 60 times a minute. This was necessary in order for the isopropyl ester, which is practically insoluble in water, to be properly mixed with the aqueous solution.

Determination of the hydrolysis was performed by mixing 0.2 and 0.05 ml, respectively, of the isopropyl ester of DL-, L- or D-phenylalanine with 2.5 ml of solution of the dry powder

**Table I.**

*pH values of buffer solutions after addition of isopropyl ester of phenylalanine and enzyme solution*

(2 % enzyme solution with 0.2 ml of ester, and 0.5 % with 0.05 ml of ester).

| 5 % glycocoll | 1 *N* HCl | 0.1 *N* HCl | 1 *N* NaOH | 0.1 *N* NaOH | Water | Enzyme solution | pH after addition of isopropyl ester of phenylalanine in quantities of | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | 0.2 ml | 0.05 ml |
| ml | ml | ml | ml | ml | ml | ml | pH | pH |
| 1.00 | — | — | 0.7 | — | 0.8 | 2.5 | 10.7 | 11.1 |
| 1.00 | — | — | 0.5 | — | 1.0 | » | 10.0 | 10.2 |
| 1.00 | — | — | 0.4 | — | 1.1 | » | 9.7 | 9.9 |
| 1.00 | — | — | 0.2 | — | 1.3 | » | 9.2 | 9.3 |
| 1.00 | — | — | 0.1 | — | 1.4 | » | 8.9 | 9.0 |
| 1.00 | — | — | — | 0.5 | 1.0 | » | — | 8.7 |
| 1.00 | — | — | — | — | 1.5 | » | 8.2 | 8.2 |
| 1.00 | — | 0.5 | — | — | 1.0 | » | — | 7.6 |
| 1.00 | — | 1.5 | — | — | 0.5 | » | — | 7.0 |
| 1.00 | 0.4 | — | — | — | 1.1 | » | 7.4 | |
| 1.00 | 0.8 | — | — | — | 0.7 | » | 6.3 | |

from the duodenal mucosa, and 2.5 ml of buffer solution. When the experiment was performed with 0.05 ml of ester, this was obtained by adding 0.2 ml of a 25 per cent solution of the ester in absolute alcohol. The strength of the enzyme solutions can be inferred from the relevant experiments.

The buffer system used was 5 per cent glycocoll — 1 *N* or 0.1 *N* sodium hydroxide or 5 per cent glycocoll — 1 *N* and 0.1 *N* HCl, respectively. One ml of 5 per cent glycocoll solution was always used; sodium hydroxide or hydrochloric acid was added, as well as distilled water, to make a total volume of 2.5 ml. The ester and the intestinal enzyme solution were then added. Three tubes containing the same quantity were always taken at each determination, and the quantity of unhydrolyzed ester at the end of the reaction was determined in one of them. In the other two, the pH was determined before and after the end of the reaction, respectively. In addition, determinations were made in each experimental series of the quantity of unhydrolyzed ester in samples with only the buffer and enzymes, and in samples with the buffer, enzymes and ester, before hydrolysis, *i. e.*, immediately after mixing of the substances in question.

*Determination of the unhydrolyzed ester* was performed as follows. To the mixture of the buffer, wholly or partially hydrolyzed ester and enzymes, were added a few drops of phenolphthalein solution (1 per cent in absolute alcohol) and a sufficient quantity of 1 *N* NaOH to produce a distinctly red colour. Ten ml of ether were then added and the mixture was transferred, after shaking, to a separating funnel; the water phase

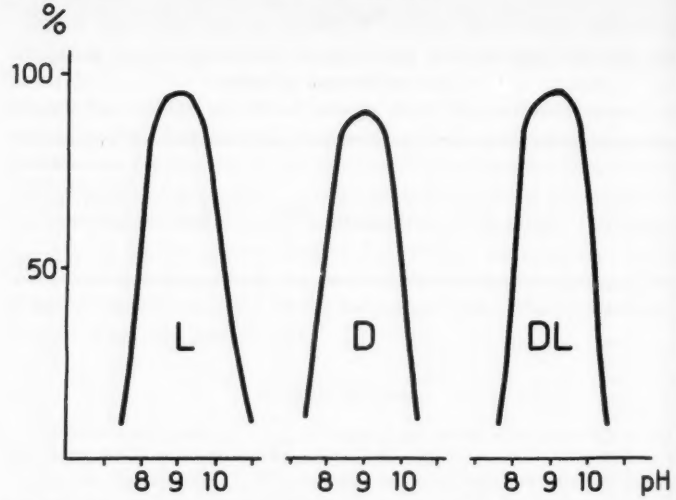


Fig. 2. *Hydrolysis of the isopropyl ester of L-, D- and DL-phenylalanine with enzymes from the duodenal mucosa.* The quantities used were 0.2 ml of ester and a 2 % enzyme solution, and 0.05 ml of ester and a 0.5 % enzyme solution, respectively. The curves represent the mean values of these experiments, which gave the same results. The ordinate shows the degree of hydrolysis in per cent, and the abscissa the pH at which the reaction took place.

was returned to the reaction tube, whereas the ether with the isopropyl ester of phenylalanine dissolved in it was transferred to another separating funnel. The water phase was again extracted with 10 ml of ether, which were added to the earlier ether extract. To the separating funnel with the two combined ether extracts were added 20 ml of 0.1 *N* HCl, and the water phase was separated off after shaking. Thereafter, 5 drops of bromophenol blue solution (0.1 per cent in 50 per cent alcohol) were added, and the solution was titrated with 0.1 *N* NaOH; the quantity of unhydrolyzed isopropyl ester could then be calculated on this basis.

*Determination of the pH optimum at 37° C* was performed with the isopropyl ester of DL-, D- and L-phenylalanine; the enzyme solution in a concentration of 2 per cent of the dry powder from the duodenal mucosa was used in experiments with 0.2 ml of the ester. Corresponding experiments were made with 0.05 ml of the ester (0.2 ml of 25 per cent ester solution in absolute alcohol); a 0.5 per cent enzyme solution was then used. The different buffer solutions used are shown in Table I, in which the pH value after addition of 0.2 and 0.05 ml, respectively, of the ester is also recorded.

The buffer solution, the ester and the enzyme solution were heated to 37° C before starting the reaction. Exactly 30 minutes after addition of the intestinal enzyme solution to the buffer and ester solution, the

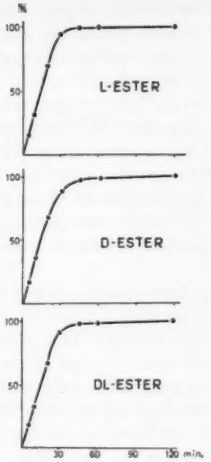


**Fig. 3.** *Relation between time and degree of hydrolysis of the isopropyl ester of L-, D- and DL-phenylalanine, at 37° C, with enzymes from the duodenal mucosa.* The experiments were performed at pH 8.9 (1 ml of 5 % glycocoll, 0.1 ml of 1 *N* NaOH and 1.4 ml of distilled water), 2.5 ml of 2 % enzyme solution and 0.2 ml of ester. The reaction temperature was 37° C. The ordinate shows the degree of hydrolysis in per cent, and the abscissa the time in min. for the reaction.

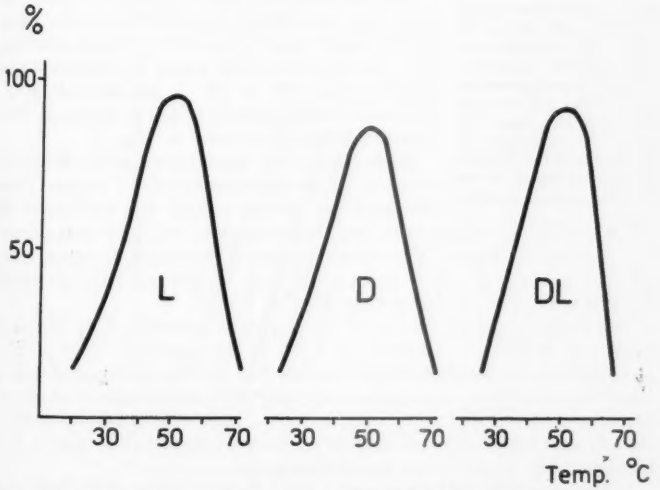


**Fig. 4.** *Relation between temperature and degree of hydrolysis of the isopropyl ester of L-, D- and DL-phenylalanine with enzymes from the duodenal mucosa.* The experiments were performed with 0.2 ml of ester at pH 8.9 (1 ml of 5 % glycocoll, 0.1 ml of 1 *N* NaOH and 1.4 ml of distilled water), using 2.5 ml of 2 % enzyme solution. The reaction time was 20 min. The ordinate shows the degree of hydrolysis in per cent, and the abscissa the temperature in ° C.

reaction was interrupted by the addition of 1 *N* NaOH and phenolphthalein; analysis of the unhydrolyzed ester was then carried out at

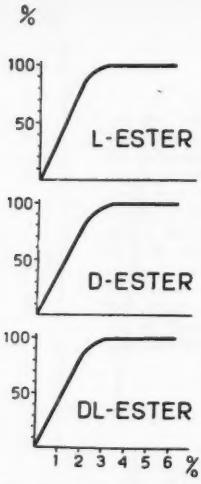


Fig. 5. *Hydrolysis of the isopropyl ester of L-, D- and DL-phenylalanine with different concentrations of a duodenal mucosa extract.* The pH was 8.9. The ordinate shows the degree of hydrolysis in per cent, and the abscissa the concentration of the duodenal mucosa powder.

once. The degree of hydrolysis in per cent was calculated from the values obtained, and the curves shown in Fig. 2 were drawn. The results were the same with 0.2 and with 0.05 ml of ester, respectively. The determinations were made with two concentrations for the following reason. When 0.2 ml was used, a shift in the pH was observed in the course of the reaction which might have affected interpretation of the result. When the quantity of ester was decreased to 0.05 ml, this shift in the pH was considerably less marked.

A study of the *relation between degree of hydrolysis and time* was carried out at 37° C with a buffer solution (1 ml of 5 per cent glycocoll, 0.1 ml of 1 *N* NaOH and 1.4 ml of distilled water) at pH 8.9, this being the optimal pH for the enzymatic reaction, and a 2 per cent enzyme solution. The results with 0.2 ml of the DL, L and D ester can be inferred from Fig. 3.

Determination of the *temperature maximum* took place in a similar way, using the aforementioned buffer and 2 per cent enzyme solution. The investigation was made at temperatures ranging from 26° to 72° C, at intervals of 2°. The duration of hydrolysis was 20 minutes. The results obtained are seen in Fig. 4.

*Hydrolysis of the isopropyl esters of phenylalanine with different concentrations of enzymes from the duodenal mucosa extract* was performed at pH 8.9 with the aforementioned buffer solution, at 37° C and a reaction time of 30 minutes. The concentrations of the enzyme solution were 0.0, 0.5, 1.0, 1.5, 2.5, 3.0, 3.5, 4.5, 5.0 and 6.0 per cent, the quantity being 2.5 ml. The results are recorded in Fig. 5.

The *acid-neutralizing ability* of the ester was determined by titration with 1.00 *N* HCl of a mixture of 1 ml of the isopropyl ester of DL-phenylalanine in 30 ml of distilled water. The pH was determined with a glass electrode. Since the ester is extremely difficult to dissolve in water, titration took place with stirring, and after each small addition of HCl, the pH was repeatedly determined until equilibrium in the system had occurred. Fig. 6 shows the curve obtained.

Determinations of the *toxicity* of the isopropyl ester of D-, DL- and L-phenylalanine were made on growing male rats weighing from 56 to 114 g. Growing rats were used in order to permit comparison with the toxicity earlier observed in growth experiments. The ester was instilled into the stomach through a tube in the following quantities: 5, 8, 10, 12, 13.5 and 16 ml/kg of body weight of the DL ester; 5, 6, 8, 11 and 15 ml/kg of the D ester, and 8, 13, 17, 18 and 20 ml/kg of the L ester. The different quantities were given to groups of 10 animals each. Calculation

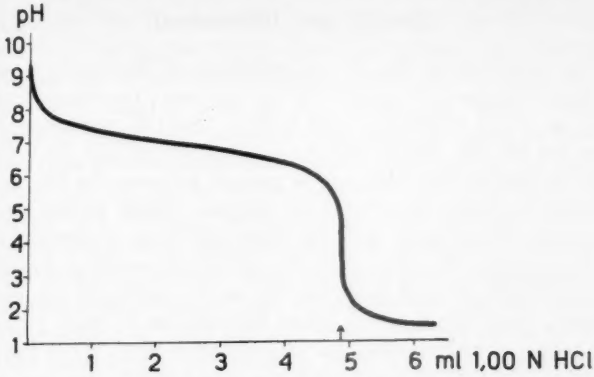


Fig. 6. *Titration curve for the isopropyl ester of DL-phenylalanine with 1.00 N HCl.* The determination was made by titration of 1.00 ml of the ester suspended in 30 ml of distilled water with a glass electrode.

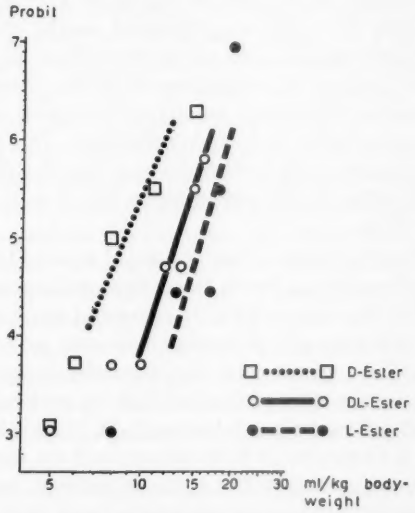


Fig. 7. *Determination of $LD_{50}$ for the isopropyl ester of D-, DL- and L-phenylalanine in rats.* Each dose was given to a group of 10 male rats. The ester was instilled into the stomach through a tube. The ordinate shows the probit for the percentage of dead animals, and the abscissa the dose administered.

of $LD_{50}$ was made with the procedure given by MILLER and TAINTER (1944). The results are seen in Fig. 7.

## Results and Discussion.

It is evident from Fig. 2 that hydrolysis of the isopropyl esters of phenylalanine with intestinal enzymes has a pronounced maximum at a pH of about 9 (8.7 to 9.2). This pH maximum is the same for the DL, the L and the D form. It can also be inferred that no appreciable difference is present between the DL, L and D ester as to the activity of the enzyme. These results already show that the enzymes split the isopropyl esters of phenylalanine symmetrically. The fact that the intestinal enzymes hydrolyze the isopropyl esters of phenylalanine at the same rate can also be inferred from Fig. 3, which shows the relation between time and the degree of hydrolysis of the DL, D and L ester. When the concentration of enzyme was varied, the corresponding changes in the degree of hydrolysis were similar in the isomers and in the racemic form of the phenylalanine ester (Fig. 5).

Investigation of the temperature optimum of hydrolysis showed, surprisingly enough, that it was as high as 50° C (Fig. 4), and also that the activity of hydrolysis underwent similar changes in the isomers and in the racemic form of the ester. Thus, all these experiments showed that enzymes are present in the intestinal mucosa which, in contrast to certain pancreatic enzymes, split the isopropyl ester of both L- and D-phenylalanine. This probably explains why rats grow well irrespectively of whether they are given the isopropyl ester of phenylalanine in the D or in the L form (WRETLIND 1954).

The acid-binding ability of the isopropyl ester of DL-phenylalanine can be inferred from Fig. 6. It can be seen from the curve that, at about pH 4, the ester is entirely converted into the hydrochloride, and that 4.9 ml of 1 *N* hydrochloric acid are required per 1 ml of ester. This agrees with the theoretical calculation that can be made on the basis of the fact that the molecular weight of the isopropyl ester of phenylalanine ($C_{12}H_{17}O_2N$) is 207.25, which implies that 4.97 ml of 1 *N* hydrochloric acid are required per ml of ester (sp. wt. = 1.028) for it to be entirely converted into hydrochloride. It is also evident from the curve that, in saturated aqueous solution, the isopropyl ester of DL-phenylalanine gives a pH of 9.2. Its buffering ability is poor over pH 7.5. The strongest buffering lies between pH 7.5 and 6.5.

The toxicity of the isopropyl esters of phenylalanine in rats is shown in Fig. 7. $LD_{50}$ is calculated to be $8.7 \pm 0.6$ ml/kg of body

weight for the D ester, 13.0 ± 0.6 ml/kg for the DL ester, and 16.0 ± 0.8 ml/kg for the L ester. The animals died between 45 minutes and 12 hours after administration. Before death occurred, they were lethargic and lay in a somnolent state. Death was caused by respiratory paralysis. The heart continued to beat for a short time after respiration had ceased and cyanosis had appeared. At autopsy, the pyloric portion of the stomach was haemorrhagic and dark red in colour. Similar changes were observed in the ileum and its mesentery. The corresponding portion of intestine was filled with reddish, thin fluid, probably due to an admixture of blood from the intestinal wall by leakage through the capillaries. Extravasation of blood had also occurred round the blood vessels in the mesentery of the ileum. — These isopropyl esters have also been found to produce a local irritating effect on the stomach when given to dogs in relatively small doses (1—2 ml) through a stomach tube. A fairly high incidence of vomiting was noted in these cases.

The remarkable feature in this case is the great difference between the toxicity of the D ester ($LD_{50} = 8.7 \pm 0.6$ ml/kg) and that of the L ester ($LD_{50} = 16 \pm 0.8$ ml/kg). This difference is statistically significant. As could be expected, $LD_{50}$ for the DL ester lay between these values ($LD_{50} = 13.0 \pm 0.6$ ml/kg). Earlier investigations both on the acute toxicity (Gullino, Winitz, Birnbaum, Cornfield, Ostey and Greenstein 1955) and the chronic toxicity (Wretlind 1952) have shown that the D form of phenylalanine is less toxic than the L form. This indicates that the toxicity of the isopropyl esters of phenylalanine is not brought about by the influence of the phenylalanine obtained after hydrolysis of the esters since, in this case, the L ester should be more toxic. It is, however, fully evident from the experiments that, from the quantitative viewpoint, there is an appreciable difference between the effect on the animals of the D and of the L ester. No qualitative differences were observed in these toxicity determinations.

The experiments hitherto made indicate that amino acid esters of the type in question would be suitable, in view of their relatively low toxicity and acid-neutralizing effect, for producing neutralization of the hydrochloric acid in the stomach in such conditions as hyperacidity. It is evident from the present investigations that the esters are hydrolyzed in the duodenum, whereby the chloride ions are released and absorbed. Other constituents of the esters, *i. e.*,

isopropyl alcohol and phenylalanine, are metabolized or are included in the utilization of protein. Consequently, an acid-neutralizing effect can be achieved without a change in the acid-base balance of the body.

## Summary.

Earlier investigations have shown that the isopropyl ester of DL-phenylalanine is hydrolyzed asymmetrically by pancreatic enzymes. It is also known that the isopropyl esters of both D- and L-phenylalanine produce growth in rats, thus implying that they must both be hydrolyzed in the organism. This is shown in the present investigation to depend on the fact that enzymes are present in the duodenal mucosa which hydrolyze the isopropyl esters of D-, L-, and DL-phenylalanine equally rapidly. A closer study has been made of this enzymatic hydrolysis of the esters. It is found that the pH optimum for enzymatic hydrolysis lies at pH 8.7 to 9.2 for the L, the D and the DL ester.

The temperature optimum is found to be 50° C for all three esters.

With a change in the time, pH and enzyme concentration, the rate of hydrolysis by the enzymes in question undergoes similar changes in the isopropyl ester of L-, of D-, and of DL-phenylalanine.

The toxicity of the isopropyl esters of phenylalanine was determined in growing rats. The $LD_{50}$ was found to be $8.7 \pm 0.6$ ml/kg of body weight for the D ester, $16.0 \pm 0.8$ ml/kg for the L ester, and $13.0 \pm 0.6$ ml/kg for the DL ester. The cause of death was respiratory paralysis. Pathologic changes of haemorrhagic type were observed in the pyloric portion of the ventricle and in the ileum.

The acid-neutralizing ability of the ester was determined, and found to amount to 4.9 ml of 1 *N* hydrochloric acid per ml of ester.

The possibility is suggested of using such amino acid esters for neutralization of the hydrochloric acid of the stomach.

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# Central Control of Heat Loss Mechanisms in the Goat.

By

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Since MAGOUN, HARRISON, BROBECK and RANSON (1938) showed that local heating of parts of the preoptic area and hypothalamus of the cat causes polypneic panting and often the appearance of sweat on the foot pads it has been thought generally that structures in this area are activated by a rise in body temperature — an activation which, in the overheated normal animal, leads to mobilisation of the several heat loss mechanisms.

The thermosensitivity of this part of the brain stem was further demonstrated by FOLKOW, STRÖM and UVNÄS (1949) and by STRÖM (1950 a and b) using vasodilatation in the cutaneous vessels of the limbs as an index of activation. The thermosensitive area was shown by ELIASSON and STRÖM (1950) to lie mainly between the anterior commissure and the optic chiasma. But electrical stimulation of the same area in anesthetized cats was found to cause cutaneous vasoconstriction, often with post-stimulatory vasodilatation. Simultaneous cutaneous vasoconstriction and polypneic panting were sometimes seen during electrical stimulation of both anterior and posterior hypothalamus (STRÖM 1950 b), but simultaneous cutaneous vasodilatation and panting were not seen with either electrical or thermal stimulation.

Thus despite clear evidence that the anterior brain stem contains neurons especially sensitive to warming the question as to

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whether a unitary "heat loss centre" exists in this area, capable of controlling the various heat dissipation mechanisms, remains unanswered, as does that regarding whether warming excites or inhibits the neurons affected (STRÖM 1950 b, GRANT 1951).

In experiments where electrical stimulations within the preoptic area and the anterior hypothalamus were made in unanesthetized goats in order to map out the region concerned with water intake (ANDERSSON and MCCANN 1955) it was observed that the stimulation of a very restricted area just ventral to the anterior commissure caused vigorous polypnea. Since the use of an unanesthetized experimental animal with a relatively large brain such as the goat, together with strictly localized electrical stimulation, offered several advantages over methods used previously the experiments described below were undertaken in order to define the anatomical position of the "panting centre" more precisely and to determine whether stimulation of this area affects other functions, especially those concerned with thermoregulation.

## Methods.

Ten fullgrown, female goats, 4 of them lactating, were used for the experiments. For electrical stimulations HESS' technique (1932, 1949), somewhat modified for the use in goats (ANDERSSON 1951) was used. Three electrodes could be stimulated either simultaneously or individually at a given depth of the brain with this technique. The parameters of stimulation were generally 0.5 to 1.5 V and 50 cycles/sec. Pulses were of 7 msec. duration, slightly damped square waves with the DC component removed.

The animals were either unrestrained or, during recording procedures, kept in a Pavlov stand and the rectal temperature was continuously recorded by help of a thermocouple connected to an "Ellab" thermometer. In the same way the skin temperature of the ear could be recorded by a thermocouple attached to the tip of the ear. A pneumograph around the chest permitted a kymograph record of respiration rate to be obtained. When desired, the diuresis of the animals was continuously recorded with the aid of a "Dowse's" self-retaining catheter, and in the lactating goats the teats were cannulated during the experiments in order to make it possible to observe milk ejection. If arterial blood pressure was recorded this was done directly in the mammary artery under sacral anesthesia.

For a study of the effect of high environmental temperature the goats were placed in an electrically heated chamber where the temperature was kept constant at a desired level by means of a thermostat. A low environmental temperature, on the other hand, was obtained

by placing the animals in a freezing room at about — 5° C, and a rapid fall of the body temperature of the goats was produced by giving cold water by stomach tube into the rumen.

*Chemical methods.* Blood glucose was determined by the method of FOLIN and WU (1930). Sodium and potassium analyses in urine were made using the EEL flame photometer. Urine chloride was determined according to the Volhard method (PETERS and VAN SLYKE 1932).

*Histological technique.* After killing the animals by decapitation, the heads were perfused with Bouin's fixative, and after fixation and paraffine imbedding, the diencephalon and preoptic area were serially sectioned and stained with cresyl violet. The localization of the effective points of stimulation was facilitated by making small electrocoagulations.

## Results.

### A. Exposure of the Goat to Heat and Cold.

As it appeared to be of interest for the interpretation of the stimulatory experiments described in the following and as no previous study of the effect of an exposure of the goat to either heat or cold seemed to have been reported, four of the experimental animals were on several occasions and for different lengths of time placed in a constant temperature chamber, where the temperature was kept at 50° C, or in a freezing room with a temperature of about — 5° C. The rate of respiration and the rectal temperature were continuously recorded, and in addition to that the effect of the high environmental temperature on the course of the water diuresis was evaluated in two of the animals, which had been hydrated with a load of warm water of 6 to 7 liters given by stomach tube into the rumen 1 1/2 to 2 hours before the exposure to heat.

*The effect of exposure to heat.* The rate of respiration of the goats before they were led in from room temperature to the heated chamber varied between 30 and 40 breaths per minute. After about 5 minutes' exposure to a temperature of 50° C the respiration rate started to increase gradually and the animals began panting continuously after 15 to 20 minutes with a respiration rate of 200 to 260 per minute. Before the onset of continuous panting the rectal temperature of the goats had risen 0.4 to 0.7° C. When the animals were taken out into room temperature again the polypnea continued for different lengths of time. If the exposure to heat had not exceeded 30 minutes the respiration rate came back to normal within 5 minutes after the goats were

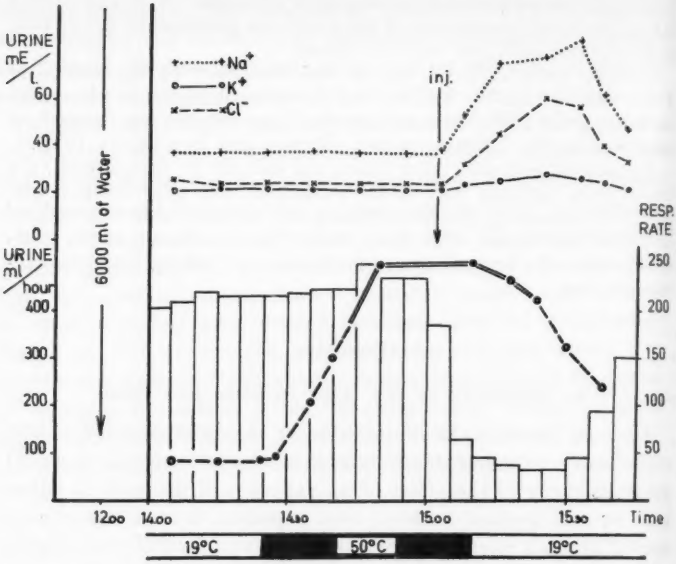


Fig. 1. A graph showing the respiration rate, the course of a water diuresis (open columns) and the urinary concentrations of electrolytes in a goat before, during and after exposure to an environmental temperature of 50° C.
Double lining of respiration curve indicates duration of panting.
The arrow marked *inj.* indicates the intravenous injection of 8 milliunits of "Pituitrin".

replaced in room temperature, whereas in the hydration experiments where the exposure to heat had been 40 to 50 minutes, causing a more than 1° C rise in rectal temperature, the panting continued 10 to 15 minutes after the animals were taken out into room temperature (19° C).

The exposure to heat and the onset of polypnea and panting were in no case found to change the course of the water diuresis of the animals or their urinary excretion of electrolytes although the sensitivity of the kidneys to small amounts of "Pituitrin" (5—10 milliunits given intravenously) was the same in the hydrated panting animal, standing in the heat chamber, as in the same animal, hydrated, but not exposed to heat. Fig. 1 shows records of urine flow, urinary concentrations of electrolytes and respiration rate of a hydrated goat exposed to an environmental temperature of 50° C during 45 minutes.

*The effect of exposure to cold.* One to four minutes after the goats were taken from room temperature to an environmental temperature of — 5° C they started to shiver, a shivering which generally became more and more vigorous the longer the exposure to cold lasted. The rectal temperature of the animals started to rise and had after one and a half hours in the cold environment in most cases reached a level 1 to 1.5° C higher than it had been when the goats were taken into the cold room. After this time in the cold environment the animals were again taken out into room temperature. Here they continued to shiver for about 5 minutes and within half an hour the rectal temperature had dropped to the initial value.

### B. Electrical Stimulations of the "Heat Loss Centre" Ventral to the Anterior Commissure.

*1) Stimulations in goats kept at room temperature (19° C).* The animals did not seem to be much disturbed by the electrical stimulations mobilising heat loss mechanisms and in most cases polypnea and panting were the only visible effects of the stimulations, although side effects were occasionally observed when stimulating in the periphery of the reactive zone. When the environmental temperature was 18 to 20° C and the rectal temperature of the animals normal for this external temperature (39.0 to 39.5° C) the polypnea started almost immediately after the stimulus was applied and panting generally began after a time of latency of about 5 seconds. The rate of respiration increased from a prestimulation level of about 40 per minute to 200 to 300 per minute and the animals opened their mouths and widened their nostrils when the panting became vigorous. The polypnea always continued with about the same intensity throughout the period of electrical stimulation even when this was prolonged to 5 minutes or more. After the stimulation was discontinued the goats might go on panting for one to two minutes and the polypnea persist, although gradually declining, up to 10 minutes after the end of electrical stimulation. Simultaneously with the polypnea there was a vasodilatation in the ears as recorded by increased ear surface temperature. But this increase did not commence until $^1/_2$ to 1 $^1/_2$ minutes after the onset of electrical stimulation. Thus, if the period of stimulation was shorter than 1 $^1/_2$ minute, the highest level of the ear surface

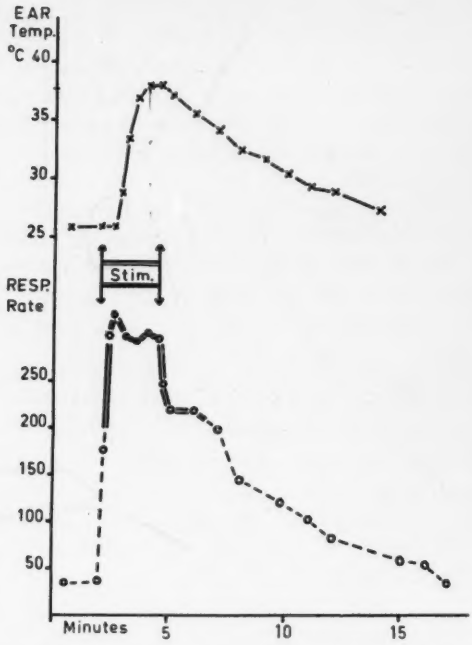


Fig. 2. The effect of a 2 minute period of electrical stimulation (1 V, 50 cycles/sec.) of the "heat loss centre" ventral to the anterior commissure on respiration rate and ear surface temperature in a goat kept in room temperature (19° C). Double lining of respiration curve indicates duration of panting.
*Stim.:* Period of electrical stimulation.

temperature was not reached until after cessation of stimulation. The ear temperature then gradually declined and reached the prestimulation level 5 to 10 minutes after the end of electrical stimulation. Fig. 2 shows the typical changes in respiration rate and surface temperature in an animal where the "heat loss centre" was stimulated at room temperature.

Under these conditions it was possible to lower the rectal temperature of the goats 0.5 to 1° C by continuing the electrical stimulation for 10 to 15 minutes.

The electrical stimulations causing polypnea and ear vasodilatation did not produce any significant change in the blood sugar level of the animals and had no effect on the heart rate or the arterial blood pressure except for a short initial rise of arterial

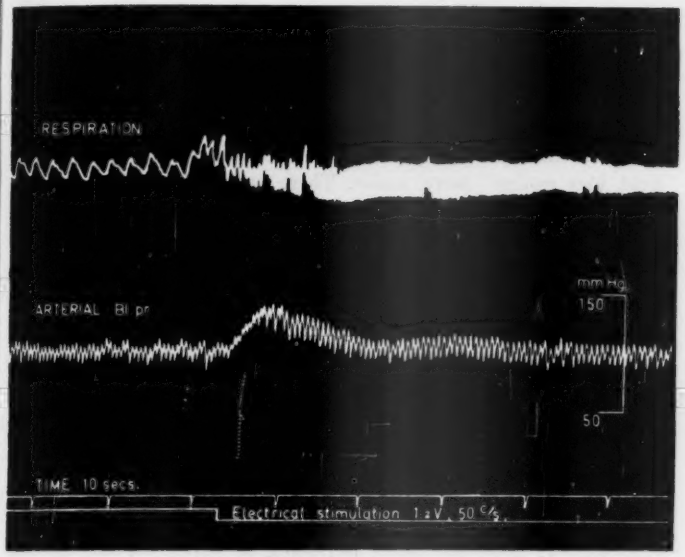


Fig. 3. A record of respiration rate and blood pressure in the mammary artery of a goat before and during electrical stimulation of the "heat loss centre".

pressure just at the beginning of the electrical stimulations (Fig. 3). This initial rise of pressure was also obtained when stimulating points anterior and posterior to the "heat loss centre" and, further, an increase of the frequency of stimulation to 300 cycles/sec. at a point where the stimulation with 50 cycles/sec. resulted in marked polypnea caused the disappearance of the polypneic effect but did not abolish the initial rise of blood pressure seen during the stimulation.

These stimulations causing a mobilisation of heat loss mechanisms were not found to cause any inhibition of water diuresis of hydrated animals or milk ejection in lactating goats with the exception of stimulations at the ventro-caudal border of the reactive area (see below).

*2) Stimulations in goats kept at an environmental temperature of about —5° C.* After an electrode had been placed in the "heat loss centre" with the animal kept in room temperature and a few preliminary stimulations had been made to determine the strength of stimulation needed to produce a certain degree of

polypnea and vasodilatation in the ears the animals were taken into the freezing room and kept there at an environmental temperature of about — 5° C. As in previously tested animals shivering soon started and the same rise in rectal temperature was observed. When the animals had been in the cold environment for about half an hour renewed electrical stimulations of the "heat loss centre" were made. These stimulations stopped the shivering immediately and completely and, as previously, caused polypnea and vasodilatation of the ears, but the strength of stimulation had to be approximately doubled to obtain the same degree of polypnea as that seen in room temperature about half an hour earlier. Arrest of shivering could not be obtained from stimulations at points 2 mm anterior or posterior to the reactive area ventral to the anterior commissure even when using three times stronger stimulation.

The first two or three 2 minute periods of stimulation of the "heat loss centre" in the cold environment caused an inhibition of shivering lasting 2 to 4 minutes after the end of electrical stimulation and a rise of ear surface temperature lasting somewhat longer. Here too some polypnea persisted for one or two minutes after the end of electrical stimulation. The after effects for polypnea and ear vasodilatation were, however, considerably shorter than after similar stimulations made with the animals kept at room temperature.

Repeated stimulations of the "heat loss centre" with the animals in the cold environment rapidly lowered their rectal temperatures. A fall of 1 $^1/_2$° C could thus easily be obtained (Fig. 4). When the rectal temperature had fallen that much the strength of stimulus needed to cause a vasodilatation of the ears was raised considerably and the inhibition of shivering did not last more than $^1/_2$ minute after the end of stimulation. In fact, a weak electrical stimulation, which in room temperature had produced a polypnea of about 150 breaths per minute did not now cause any increase of respiration rate and did not any longer cause vasodilatation of the ears or inhibit shivering, although the same strength of stimulus had completely inhibited shivering during the first stimulations in the cold environment, when the rectal temperature of the animals was 1 $^1/_2$° C higher. Stimulations of sufficient strength to cause moderate polypnea always inhibited shivering but often caused no recordable change of the ear surface temperature.

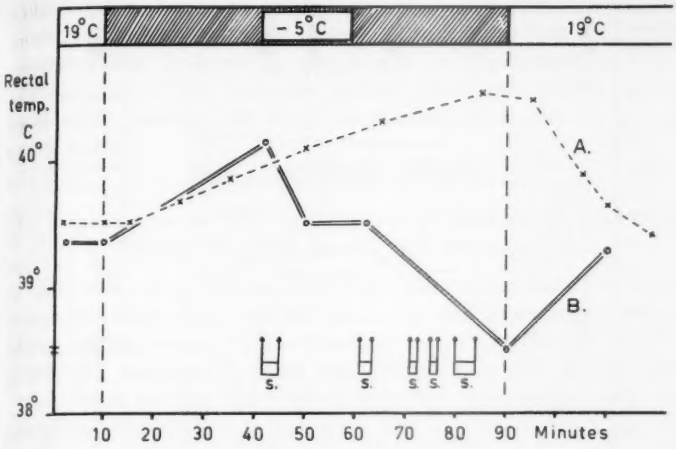


Fig. 4. Changes in rectal temperature in a normal goat during 1 ½ hours' exposure to an environmental temperature of — 5° C (curve A) compared to the changes in rectal temperature in a goat similarly exposed to cold but in which, after half an hour, repeated electrical stimulations of the "heat loss centre" were made (curve B).

S: Periods of electrical stimulation.

After the goats had been brought back to room temperature and had been there for one to two hours renewed stimulations were made and the sensitivity of the "heat loss centre" to electrical stimulation was now found to have increased towards the level it had been at before the goats were brought into the cold room.

*3) Stimulations in goats where the body temperature had been rapidly lowered.* In order to obtain a rapid fall of body temperature of the goats without changing the environmental temperature some animals were given 3.5 liters of water at a temperature of 10° C into the rumen by stomach tube. This caused an almost immediate drop in rectal temperature of 1 ½ to 2° C and an immediate onset of vigorous shivering. The shivering lasted for somewhat more than an hour and then disappeared although the rectal temperature of the animals still was 1° C lower than normal. Before this artificial lowering of the body temperature of the animals, the sensitivity of the goats to electrical stimulation of the "heat loss centre" had been tested.

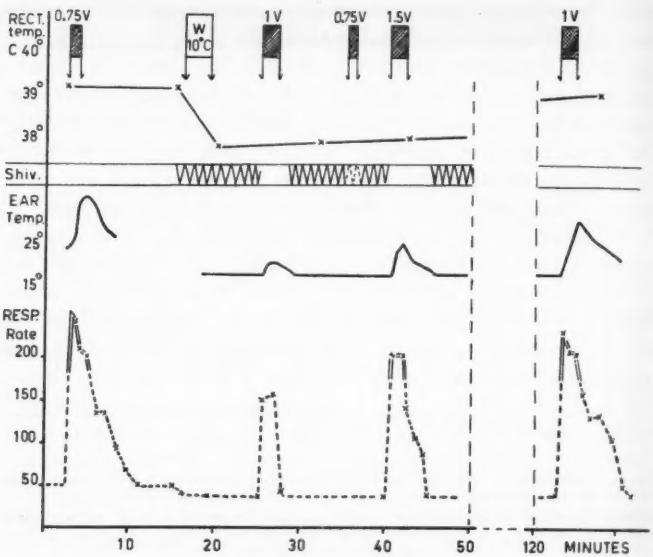


Fig. 5. The effect on respiration rate, ear surface temperature and shivering of electrical stimulations of the "heat loss centre" of a goat before and after the body temperature was lowered by giving 3.5 liters of water at 10° C by stomach tube into the rumen.

*Shiv.:* Shivering.
Double lining of respiration curve indicates duration of panting.

Similar observations were made after lowering the body temperature in this way as after lowering it by repeated electrical stimulations of the "heat loss centre" in the cold environment. Thus there was a considerable rise in the threshold for the polypneic and vasodilatator responses and a weak stimulus which before the lowering of the body temperature had caused both polypnea and vasodilatation in the ears was now ineffective in causing any change in respiration rate and ear surface temperature and did not completely inhibit shivering. Stronger stimuli, which caused polypnea, did however always completely arrest the shivering at least during the actual period of stimulation, even if no rise or a very small rise in ear temperature was seen.

When sufficient time had lapsed for the body temperature to reach normal or almost normal level the sensitivity to stimula-

tions of the "heat loss centre" was again increased towards the level it showed before the body temperature was artificially lowered. Fig. 5 shows an example of an experiment in which stimulations of the "heat loss centre" were made before, during and after lowering the body temperature of a goat by giving cold water.

### C. Localisation of Effective Points of Stimulation and Side Effects Observed During the Stimulations.

The area where electrical stimulations caused heat loss reactions was fairly restricted and is part of the larger region where local heating causes similar reactions in the cat (MAGOUN et al. 1938). In contrast to what was observed in the experiments where local heating was used as stimulus the effective area for electrical stimulation in the goat was limited to what should strictly be described as the preoptic area (Fig. 6). Cytologically this area in the goat consists of mainly small cells but in its anterior part are numerous, scattered, large neurons with densely granular cytoplasm. The discreteness of the reactive region was indicated by the fact that in one and the same animal stimulation at the middle of the three electrodes (the tips of which were 2 mm apart) might be effective, whereas three times stronger stimulations at the anterior and posterior electrode tips caused no polypnea or vasodilatation in the ears and did not inhibit shivering. The reactive zone was limited laterally by the medial border of the internal capsula and dorsally by the anterior commissure. It occupied only the dorsal half of the area between the anterior commissure and the optic chiasm. Fig. 6 shows the extent of the region on a parasagittal section and Fig. 7 the different points of stimulation projected on a horizontal section at a level about 2 mm ventral to the anterior commissure.

No notable side effects were observed when stimulating in the centre of the reactive region. But stimulations at its ventrocaudal border did cause inhibition of water diuresis of neurohypophyseal type and milk ejection in lactating animals and occasional drinking. Stimulations dorsally at the posterior border of the "heat loss centre" or further back towards the Columna fornicis descendens sometimes caused a pattern of behavior seemingly related to the sensation of smell and described previously (ANDERSSON and MCCANN 1955). No other notable effects

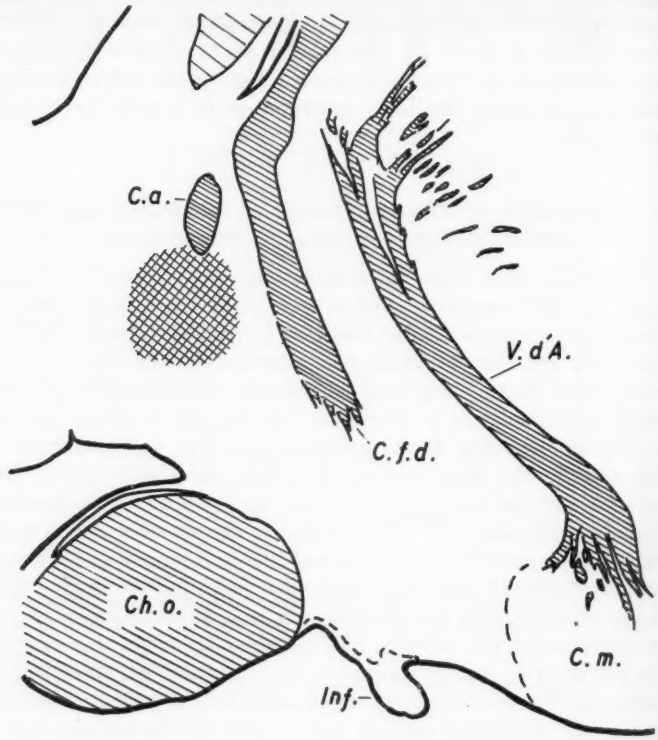


Fig. 6. A diagram of a parasagittal section through the preoptic area and the hypothalamus of the goat. The cross hatched area marks the region where electrical stimulations caused polypnea, vasodilatation in the ears and inhibition of shivering.

C. a.: Commissura anterior
C. f. d.: Columna fornicis descendens
Ch. o.: Chiasma opticum
C. m.: Corpus mammillare
Inf.: Infundibulum
V. d'A.: Tractus Vicq d'Azyr

with certainty related to the stimulations were observed when stimulating within or close to the actual region and the stimulations did not seem to "stress" the animals or cause them any visible discomfort. The absence of "stress" is confirmed by the lack of significant changes in blood sugar level.

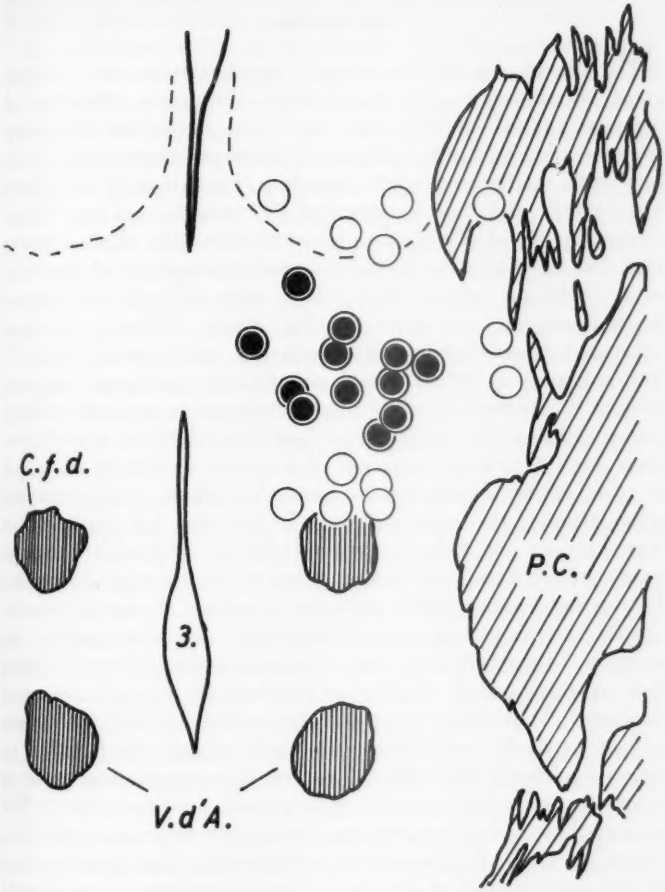


Fig. 7. A diagram of a horizontal section of the preoptic area and the hypothalamus of the goat at a level about 2 mm ventral to the anterior commissure. *Filled circles* mark points where electrical stimulation caused polypnea, vasodilatation in the ears and inhibition of shivering.
*Open circles* mark the localisation of adjacent points where electrical stimulation was ineffective in these respects.

C. f. d.: Columna fornicis descendens
P. C.: Pedunculus cerebri
V. d'A.: Tractus Vicq d'Azyr
3: Third ventricle

## Discussion.

The importance of the anterior hypothalamic and preoptic areas in relation to the mobilisation of various physiological defenses against hyperthermia has been confirmed by many workers using both stimulation and lesion-placement techniques, but much confusion persists regarding the nature of the "heat loss centre" and indeed regarding the existence of any single centre capable of effecting coordinated activation of the various heat loss mechanisms. In the classical experiments of MAGOUN et al. (1938) an increase of 3.7° C or more in local temperature seemed necessary to cause panting. STRÖM (1950 a) obtained cutaneous vasodilatation with much less local heating (0.5° C) but uniformly failed to obtain simultaneous polypneic panting despite the facilitating effect of the urethane or urethane-chloralose anesthesia used on polypnea. Results of electrical stimulation have been especially confused. MAGOUN et al. (1938) obtained a great variety of respiratory and other effects from electrical stimulation of the area responsive to warming but panting was seen only in one animal. STRÖM (1950 b) sometimes obtained panting (with cutaneous vasoconstriction) from electrical stimulation of both anterior and posterior hypothalamic areas but not consistently from any area. HESS (1948) describes panting responses obtained from the entire hypothalamus and septal region but no concentration of effective points in the preoptic area was indicated. Thus three features of the results obtained with goats are of particular significance: 1) Weak stimuli which failed to cause significant "side effects" caused vigorous panting; 2) Such responses were obtained only from a small preoptic area; 3) The stimulations caused appropriate vasomotor responses (vasodilatation in the ears, presumably an important radiating surface in the goat) instead of the inappropriate cutaneous vasoconstrictions observed by STRÖM in the cat.

It seems likely that two experimental factors contribute to these differences: a) The larger goat brain permitted specific stimulation of the "heat loss centre". b) Urethane anesthesia itself promotes cutaneous vasodilatation and polypnea in cats. The site of action of the anesthetic in this regard is unknown but the possibility that responses to stimulation of the "heat loss centre" might be greatly modified by anesthesia is clear.

Different stimulus parameters might also be an important source of difference in results.

No systematic study of all possible "side effects" of stimulations in the area giving the polypneic response has been made so that it is not possible to say that the area defined is concerned only with thermoregulatory responses, but the absence of significant blood pressure changes, effects on water diuresis, milk ejection or blood sugar in most cases suggests that the area might be of relatively "pure" thermoregulatory significance. It is of great interest that the area evidently does suppress shivering, presumably through inhibitory connections with those unknown neurons of the posterior hypothalamic "cold defence centre" excitation of which causes shivering. The area of the cat brain found by HEMINGWAY, FORGRAVE and BIRZIS (1954) to suppress shivering on electrical stimulation seems to have its most sensitive part in the dorsal preoptic region.

It is known that acute heat stress might affect renal hemodynamics (RADIGAN and ROBINSON 1949) and it has been shown that it does provoke a discharge of antidiuretic hormone from the neurohypophysis of the rat (ITOH 1954). It therefore seemed to be of interest to investigate whether this would be the case also in the goat. The fact that an acute exposure to heat strong enough to cause vigorous panting did not cause an inhibition of an established water diuresis although the sensitivity of the kidneys to antidiuretic hormone was not significantly changed strongly suggests that the "heat loss centre" in the preoptic area can be highly activated without causing the release of physiologically active amounts of antidiuretic hormone from the neurohypophysis of the hydrated goat. This conception is further supported by the observation that in most cases the electrical stimulation of the "centre" causing polypnea and peripheral vasodilatation did not inhibit an established water diuresis. However, possible effects on urine flow in the non-hydrated animal were not studied. The fact that an inhibition of water diuresis of neurohypophyseal type and milk ejection might be seen when stimulating the ventro-caudal part of the reactive region can probably be explained by a spread of the stimulus to the adjacent part of the hypothalamus which has earlier been shown (ANDERSSON and MCCANN 1955) to respond to electrical stimulation in this way.

Use of weak electrical stimuli in these tests has permitted

much closer localization of the effective area than could be expected with thermal stimuli which inevitably spread both by direct conduction and through the convective effect of the circulation. It is clear that the ventral part of the preoptic area is not involved and there is little, if any, extension into the hypothalamus proper.

Direct thermal sensitivity of the area has not yet been demonstrated in goats but in view of the exact coincidence of the anatomical area with that shown by MAGOUN et al. (1938) and by STRÖM (1950 a) to be the most sensitive to warming in the cat similar thermosensitivity in the goat is highly probable. However, since it has been shown by GRANT (1950) that the complete pattern of "heat defense" (cutaneous vasodilatation, polypnea and suppression of shivering) can be evoked in rabbits by light restraint or other "emotional" stimuli the possibility must be kept in mind that the "heat loss centre" was being stimulated only indirectly through neural pathways impinging upon it. There was rarely any behavioural indication of "emotional" disturbance in the goats during stimulation of the "centre" except occasionally during stimulation near the margins of the area, *e. g.* near the internal capsule, or with stimuli several times greater than necessary to evoke panting.

The inhibiting effect of low body temperature or exposure to cold (also noted by STRÖM, using local thermal stimulation) supports the view that a specific thermoregulatory area was being stimulated, directly or indirectly, rather than part of the motor pathway for polypneic respiration. It is unfortunately impossible to draw conclusions as to whether cold tends to inhibit the effects of direct stimulation of the "centre" through sensory pathways or through lowering of brain stem temperature. The "paradoxical" increase in rectal temperature seen on exposure to cold (often described for other species) cannot be taken to indicate that hypothalamic temperature also increased. Also the fact that ear temperature responses were more readily eliminated by exposure to cold or by low body temperature does not necessarily imply that different neurons are involved in the two responses at the preoptic level. The descending pathways mediating the respiratory and vasomotor effects presumably diverge in the diencephalon. There are certainly several lower levels at which the inhibitory effects of cold on cutaneous blood flow might be exerted, also the cutaneous vasomotor pathways are involved in

barostatic and other reflexes in addition to the thermostatic. STRÖM (1950 c) found that the cutaneous vasomotor response to hypothalamic warming could easily be inhibited by local cooling of the limb or of the blood supplying the limb and he concluded that both cutaneous sensory responses and direct effects of cold on the limb vessels were probably involved in this inhibition.

There is little likelihood that any of the thermoregulatory effects observed occurred only secondarily as a consequence of preceding changes. Thus inhibition of shivering was immediate and could not be attributed to previous cutaneous vasodilatation. Cutaneous warming (at least in the ears) always showed greater latency than polypnea. In cold animals ear dilatation might be inhibited completely and in warm animals or animals previously stimulated the ears might show full vasodilatation in the absence of polypnea. Thus onset of polypnea cannot be attributed to warming of the skin. That cutaneous vasodilatation might be secondary to polypnea seems very unlikely since the expected effect of polypneic hyperventilation is mild respiratory alkalosis, a condition tending to cause cutaneous vasoconstriction rather than dilatation.

STRÖM (1950 a) noted that it was impossible to decide, on the basis of his own and previous work, whether hypothalamic warming excites or inhibits the thermoregulatory neurons, since electrical stimulation (presumed to be excitatory) of the same area invariably caused vasoconstriction in the skin. The results in goats seemingly resolve this difficulty since they indicate strongly that both polypnea and vasodilatation are due to excitation of preoptic neurons. The vasoconstriction seen by STRÖM (1950 b) may have been due to simultaneous stimulation of vasoconstrictor neurons or fibres in the same or closely adjacent areas of the small cat brain, or possibly to disturbing effects of anesthesia. The post-stimulatory cutaneous vasodilatation (sometimes persisting for 30 minutes) seen by STRÖM may represent the persisting effects of stimulating the vasomotor neurons of the "heat loss centre" — effects which were obscured during stimulation by simultaneous stimulation of vasoconstrictor neurons. The alternative explanation of persisting post-stimulatory vasodilatations considered by STRÖM and based on the studies of BRONK, PITTS and LARABEE (1940), *i. e.* that vasodilatation was due to post-stimulatory "inhibition" (post-stimulatory depression due to summation of positive after potentials) in neurons previously

excited cannot easily be applied to the results obtained in the goat where ear vasodilatation began during stimulation and persisted, sometimes for 15 minutes or more, after cessation of stimulation. Polypnea also persisted for many minutes after termination of stimulation in some cases (see Fig. 2) and it is difficult to see how these persistent effects can be explained except on the basis of persistent after discharge at some level in supra-medullary brain stem. Such discharge might be caused by mild "injury" to the area stimulated *e. g.* by electrolytic damage, but this seems unlikely. The stimulus used contained no D.C. component (stimulating pulses were balanced by opposite "depolarizing" pulses) and the electrodes at least did not polarize. Also the much more rapid subsidence of polypnea and ear vasodilatation in animals stimulated during exposure to cold would not be expected if "injury" was the cause of stimulation. WHALEN (1952) showed that local injection of magnesium into the anterior hypothalamus excites polypnea in cats and it is conceivable that ionic or other chemical changes brought about by stimulation might account for the persistence of effects and also for the relatively long latent period preceding onset of vasodilatation especially. The fact that the different thermoregulatory effects show different latencies and different durations of "after effect" suggests that different preoptic neurons might be involved for each function but obviously the differentiation might occur at lower levels in the separate descending pathways involved.

## Summary.

1. In non-anesthetized goats, the existence of a discrete "heat loss centre" has been demonstrated by electrical stimulation of the brain stem using modified Hess technique and the effects of electrical stimulation of this "centre" have been compared with those of exposing the goat to a hot environment.

2. The "centre" occupies the dorsal half only of the area between the anterior commissure and the optic chiasma and extends laterally to the medial edge of the internal capsula. Posteriorly it probably does not extend into the hypothalamus proper.

3. Stimulation of the "centre" with damped square wave stimuli at 50 cycles per second and 0.5 to 1.5 V causes vigorous polypneic panting and cutaneous vasodilatation in the ears.

These effects are similar to those resulting from exposure to an environmental temperature of 50° C for 15—20 minutes.

4. Exposure to cold or lowering of the body temperature by administration of cold water by stomach tube increases the voltage threshold for production of comparable effects and increases the latency of the responses, sometimes abolishing the ear vasodilatation response. The shivering of cold animals is inhibited promptly by stimulation of the same "centre" and the threshold for this effect likewise raised by a fall in body temperature. Repeated stimulation of the "centre" lowered body temperature especially in animals exposed to cold.

5. The effects of stimulation show some latency and a pronounced "after effect". Cold exposure increases the latencies and decreases the duration of "after effects".

6. Stimulation of the caudo-ventral part only of the "heat loss centre" caused slight inhibition of water diuresis and milk ejection. Exposure to heat did not modify the course of water diuresis or electrolyte excretion significantly. Stimuli causing vigorous panting did not affect blood pressure, heart rate or blood sugar concentration and behavioural changes were slight or absent.

7. The area defined is thought to be a true "heat loss centre" concerned with the coordinated activation of the animal's defenses against hyperthermia.

Possible reasons for the long "after effects" of stimulating the "centre" are discussed.

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